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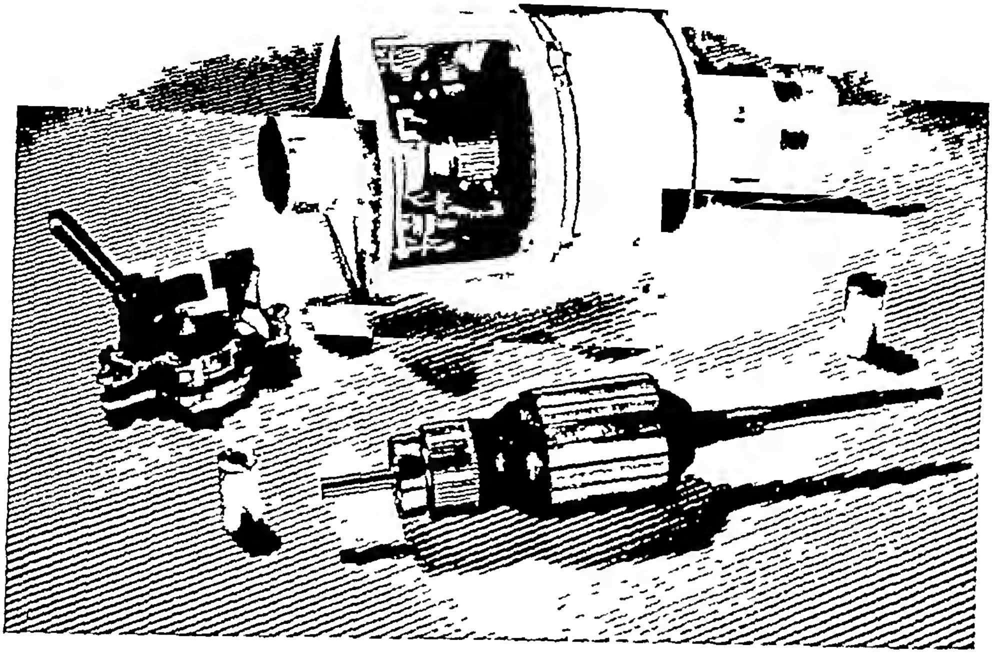

BACTERIAL DISSOCIATION

A Critical Review of a Phenomenon of Bacterial Variation[1]

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Among the long recognized aspects of variation in bacteria the phenomenon of bacterial dissociation has received particular attention The term dissociation was used by de Kruif (47) and its use extended by Hadley (86, 87), to denote the appearance, in a pure bacterial culture, of types which differ usually in several characteristics from those of the parent type, the variant type is generally sufficiently stable to maintain its characteristics over several generations, though it may give rise to still other types or, occasionally, revert to the original type In particular, the term dissociation has been applied to those changes, observed in most bacterial species, which involve a change in colony type from so-called smooth (S) to rough (R) or mucoid (M) or intermediate (I) and certain other types, usually involving not only changes in colonial morphology, but also simultaneous changes in antigenic properties, as well as various other characteristics These changes can be observed after both *in vivo* and *in vitro* growth of bacteria In a typical *in vitro* study organisms of one type, generally identified by the appearance of their colonies when viewed on solid media, are inoculated into broth, if a sample of such a broth culture is replated after a few days of growth of the originally uniform population, new colony types may be observed in addition to the original type, and the culture is then said to have dissociated

The frequently observed correlation in changes of several characteristics during this process of dissociation, and the apparently cyclic order of appearance of variant types, have led many investigators to the assumption that bacterial dissociation represents a unique and special phase in the general realm of bacterial instability Such views culminated in the proposal by Hadley (86, 87) and followers that different "phases of dissociation" represent different phases in an orderly life-cycle of bacteria Though this interpretation was not generally accepted, Hadley's publications, presented in connection with comprehensive reviews of studies on the problem of dissociation, deservedly received wide attention A decade has passed since the publication of the last of these compre-

[1] With special reference to the literature since 1935

hensive reviews and much additional information on this subject has been obtained in the meantime It, therefore, appears appropriate to review these newer data, especially in an attempt to re-evaluate the validity of various proposed concepts regarding the nature of dissociation and its relationship to problems of bacterial variation in general

I SOME GENERAL MANIFESTATIONS OF DISSOCIATION

Beginning with the studies of Arkwright (7, 8, 9) on variation in the typhoid-paratyphoid-dysentery group, the terms "smooth" (S) and "rough" (R) have become standard nomenclature of the literature on dissociation These terms were based on the occurrence of variants which produced smooth- or rough-appearing colonies, respectively, on solid media But in the early work it was also noted that this difference in colonial type was generally associated with differences in type of growth in broth, salt-sensitiveness, and changes in antigenic behavior A large amount of subsequent work established the occurrence of S and R types among the majority of bacterial species, and revealed simultaneous changes in many other characteristics which may occur during the S → R transformation (see list of changes in Hadley's 1927 review (86)) Despite the growing importance which the change in characteristics other than the colonial type achieved in the later work on dissociation, and despite the frequent absence of correlation between changes in these other characteristics and colonial form, it has remained customary to describe a type by its colony appearance, a concession to tradition which has often proved awkward

Deservedly the nature of antigenic changes during dissociation has received particular attention, and a great deal of our present knowledge on the antigenic structure of bacteria has been obtained through studies on the nature of these changes during dissociation It is, however, beyond the scope of this review to list the many interesting contributions to this problem In general, it has been recognized in many species that the S-type cells possess a type-specific polysaccharide surface antigen (the O antigen of gram-negative bacilli), usually present in the form of a definite capsule, which is absent in the R-type cells The loss of this polysaccharide capsule, in turn, accounts for several of the usual characteristics of R variants, such as loss of type-specific antigenicity, increased agglutinability by salt (through increase of lipide instead of polysaccharide components at the cell surface), altered sensitivity to bacteriophage, and, apart from certain important exceptions to be noted in detail later, a loss in virulence (*Bacillus anthracis* and the hemolytic streptococci are well-known exceptions to these general rules)

Apparently casually independent from changes in the surface antigen, and subsequent changes in characteristics dependent thereon, are other characteristics which are frequently altered during S → R variation, such as ability to sporulate (98)[2], biochemical activities, and changes in cell morphology In

[2] In the citation of examples from the literature preference will be given, where feasible, to publications which appeared after 1935, this will be done in order to accumulate references supplementing those published in earlier reviews on this subject

certain instances, however, it has been possible to correlate the latter with the characteristic changes of colonial morphology, when it was demonstrated that the morphologically different cells of the R type, for example, are associated with a differential mode of post-fission movement leading to a granular appearing colony (18, 118, 200, 201, 227) But even in the absence of morphological differences between individual S and R cells the respective cells may show strikingly different modes of development leading to the different colonial types (106)

In addition to the S and R variants, a third type of variant has been recognized in practically all species in which the phenomenon of dissociation has been studied This third variant has been labelled "mucoid" (M) on the basis of the mucoid characteristics of the colony as well as the individual cells M-type variants are usually capsulated and resemble the S type in many characteristics, but typically mucoid types with characteristics resembling the R type have also been described (31, 193), just as typical R forms with mucoid characteristics have been observed (31, 160)

Hadley (87) has termed the S, R and M types the "chief culture phases", but he and others recognized three further types occurring in a number of species, namely, *a*, "intermediate" (I) types, *b*, a G type consisting of minute colonies frequently containing filterable elements, and *c*, a D type characterized by small colonies which usually contain diphtheroid forms (for recent descriptions of small colony variants see 36, 41, 61, 85, 167, 170, 250) The intermediate types, noted quite frequently under laboratory conditions, represent apparently intermediate stages in the variation from S → R, or from M → S (*cf*, 19, 99, 166, 213) These types have been variously labelled SR, RS, MS, I, etc They may possess definite and unique colonial types, their other characteristics, however, exhibit in varying degrees those of one type (e g, S) and partly those of another one (e g, R), and they are usually quite unstable Bisset (19) has demonstrated that, as with most rough and smooth types, the colonial form adopted by these intermediate types is entirely dependent upon the physical factor of the strength of longitudinal attachment of the constituent bacilli to one another

Still another colonial type, the so-called L-type which consists of small secondary colonies containing pleuropneumonia-like organisms (120) and large spherical bodies, has lately been reported in several species (55, 56) The organisms of these colonies have been claimed to represent special phases in a reproductive cycle of bacteria

An orderly trend of variation among the three "chief culture phases" (M, S, R) has usually been claimed to be in the direction of M → S → R (87), but in some species the trend of variation has been described as S → R → M (e g, 99)

Some confusion has been created through the occasional lack of uniformity in terminology, thus some apparently mucoid types have been described as S types (*cf*, 44) This point has remained of critical importance to all those who have attempted to interpret dissociation as a part of a definite orderly scheme (cycle) and Hadley, for one, has discussed this point in some detail in his latest

review As will become apparent from the later discussion in this review, this discord in terminology may be far less critical for an understanding of dissociation than heretofore assumed

The differential stability of variant types has been widely observed under various conditions, under laboratory conditions the R type has generally proved to be the most stable one, while S and M types have exhibited various degrees of stability

Most frequently, the types found in nature are of one type only, usually the S type (typical example the typhoid species) or M type (typical example the pneumococcus species (Dawson's terminology), a notable exception the anthrax species which are normally isolated as R types), and they appear to be quite stable under conditions found in their "normal" habitat However, when grown under laboratory conditions, dissociation usually occurs, and the incidence of change has been found to be greatly dependent on the type of environment

Dissociation is more frequent in liquid media than in solid media As already mentioned, after prolonged growth of one type in any of the common fluid media employed in laboratory studies, subsequent plating on solid media will usually reveal the presence of two or more types, recognizable by differences in colonial morphology It has long been known that the addition to liquid media of antisera, small amounts of LiCl or phenol, as well as other environmental conditions to be discussed in more detail below, will greatly enhance the occurrence of dissociation On solid media, dissociation may occur in the form of secondary colonies, or it may manifest itself by the appearance of variant sectors within colonies, e g , one or more R sectors within an otherwise S colony

Isolation of variant types can generally be accomplished by picking and replating a single variant colony from a plate showing dissociation A preferable method consists of isolating a single variant cell However, due to the technical difficulties inherent in most procedures of single cell isolation, this method has found little application in the majority of studies on dissociation Nevertheless, there are a number of reports in which single cell isolation has been utilized and which describe the occurrence of dissociation in clones (a clone is the offspring of a single cell) Such reports are of particular value, because they clearly demonstrate that dissociation can occur in homogeneous populations and is not due to possible selection of variant types from an originally heterogeneous population Studies of dissociation after single cell isolation have been made with *Klebsiella pneumoniae* (108), Lactobacillus (12), *Staphylococcus aureus* (84, 103), Pneumococcus (44), *Serratia marcescens* (84), *Bacillus salmonicida* (61), *Escherichia coli* (235), *Bacillus anthracis* (230), a streptothrix (160), Salmonella (257), *Brucella abortus* (25, 26)[3], and others

II THE VARIOUS CONCEPTS OF DISSOCIATION

The foregoing brief outline of general manifestations of dissociation, most of which have been well known for many years and have been described in great

[3] The author's original work herein reviewed has been supported by the Bureau of Animal Industry, U S Dept of Agriculture, under cooperative agreement with the Regents of the University of California

detail by Hadley (86, 87), have constituted the basis for a number of concepts regarding the nature of bacterial dissociation These concepts will be cited in the following paragraphs, before additional and more recent data are presented In this manner it will be possible to eliminate more clearly some of the older concepts

The four main concepts advanced for the causative mechanism of dissociation are

1 Mutation and selection
2 Adaptation
3 "*Dauermodifikationen*"
4 Life-cycles (Cyclogeny)

1 Mutation and selection The concept of mutations, i e, spontaneous and undirected hereditary changes, entered into the bacteriological literature almost as soon as the mutation theory was advanced for higher organisms by De Vries in 1900 In 1901 and in subsequent publications (15, 16), Beijerinck suggested that phenomena of the kind which were later termed dissociation may be due to mutations Neisser (171), Massini (150), Cole and Wright (39), and Eisenberg (64, 65) were prominent among the early supporters of the mutation and selection concept in bacteriology Eisenberg, however, felt that the mutation concept alone did not suffice to explain bacterial variation and he added the concept of temporary modifications to that of mutation van Loghem (137, 138, 139, 140) extended such considerations even further, while granting the existence of mutations for certain cases of bacterial variation, he suggested additional mechanisms, which he has termed adaptation, fixation, atrophy and degeneration It may be remarked here that the factors mainly responsible for a partial, and in some cases total, rejection of the mutation concept by many, including the last named workers, were the rapidity with which a bacterial population may change its characteristics, the apparently directing influence of environmental conditions, the apparent instability of variants or the frequency of variation, the seemingly orderly succession of changes, and the frequent occurrence of reverse changes To what extent such skepticism towards the mutation concept was justified in the light of more recent knowledge will be discussed in one of the following sections Suffice it to state here that such terms as dissociation and variants became common usage, because in the absence of convincing evidence on the existence and role of mutations in bacterial variation the use of these terms was sufficiently vague to avoid connotation of the underlying mechanism of variation However, in recent years the mutation concept has attained new prominence, the work supporting it will be reviewed in a separate section

2 Adaptation Some workers entirely rejected the applicability of the mutation concept to bacteria and endowed them with a hypothetical inherent potential of sufficient flexibility to permit bacteria to adapt themselves directly to a multitude of environmental conditions Seppilli (215), for example, advocated that dissociation represents an aspect of the inherent capacity of facultative parasites to adapt their vital activities to the host, placing at one extreme the S-phase culture with essentially "animal characteristics", and at the other extreme the R-phase culture with essentially "vegetative characteristics" Sim-

ilarly, Manwaring (148) has talked about the "Lamarckian world of bacteriology" (Cockerell (38) subsequently published a critical reply to this particular argument.) Despite considerable evidence which refutes the existence of any orderly adaptation in most instances of bacterial variation (see below) the idea of direct adaptation has persisted in the bacteriological literature and terms such as "adapted" and "acquired" can still be found in many descriptive studies which are wholly unconcerned with the underlying mechanism of variation.

3. *"Dauermodifikationen"*. Another concept, introduced by Jollos (115, 115a), is that of *"Dauermodifikationen"* (lasting modifications). These are adaptive modifications induced by the environment which, without effect on any inherent factors, will persist for a considerable time after removal of the environmental condition which induced the change. While the experimental work on which this concept is based was done with protozoa, Jollos later proposed its application to bacteria. Kolbmüller (121) developed this idea even further and proposed an entirely new terminology to express this mechanism. Jackson's speculation on temporary catalyst modifications as causes for dissociation (111) were closely related to these ideas. Subsequent criticism directed against the concept of *"Dauermodifikationen"* in bacteria will be discussed below.

4. *Life-cycles* (*Cyclogeny*). The most widely discussed of all concepts is probably the "cyclogenic" or ontogenic theory advanced by Enderlein (66), Mellon (157, 158, 160) and others, which found its greatest supporter in Hadley. The origin of ontogenic concepts may actually be traced back to the bacteriological work of the 1870's, when the apparent fragmentation (i.e., "dissociation") of long filamentous cell structures into shorter bodies, and subsequent reversal of this change under proper conditions, was first observed. However, the later work did not only extend the idea of dissociation beyond this simple cellular change to the numerous variational aspects now grouped under this label, gathering to itself a major portion of the problems of bacterial variability, but also extended the ontogenic or cyclogenic concept to all these phenomena. For a detailed discussion of this particular interpretation of dissociation the reader is referred to the writings by Hadley (86, 87, 90) and a more recent paper by Mellon (160). In general, the cyclogenic or ontogenic theory underwrites the polyphasic potencies of the bacterial cell and maintains that the "chief culture phases" (M, S, R) are stages in an orderly life-cycle in which the expression of each stage is dependent on the environmental conditions. Thus, Hadley stated (88) "that each culture phase represents a stage in the development of the bacterial individual, whose span of life extends from the gonidium (or similar reproductive entity) to the reproductively mature rough-phase culture. On these grounds, the bacterial individuum should not be conceived of as a single cell, but as the entire range of successive culture development from gonidium to mature form."

III. ELIMINATION OF SOME OLDER CONCEPTS AND PRESENTATION OF NEW DATA FAVORING THE MUTATION-SELECTION THEORY

As hinted in the foregoing brief presentation of the main concepts regarding the nature of dissociation, the literature of recent years has produced much fac-

tual evidence against the concepts of life-cycles, adaptation, and "*Dauermodifikationen*"

1 *Evidence against the cyclogenic concept* To begin with the evidence against cyclogenic interpretations, a number of valuable contributions concerned with critical considerations opposing this type of interpretation are contained in publications by Mayer (151), Reed (193, 194), Dubos (60), Lindegren (134), Humphries (108), Reiman (195, 196), and Morton (168) The main evidence against cyclogenic interpretations is twofold *a*, the frequent absence of orderly succession of "culture phases", and *b*, the occurrence of independent changes of characteristics

The first mentioned evidence is illustrated by studies on pneumococci (20), *E coli* (235), and *B abortus* (99), where, after culturing of an intermediate variant, S as well as R types were observed, still other studies demonstrated variation from one variant in either direction (M → R, or R → S, or S → M), e g, in *S marcescens* (193), Pneumococcus (20, 43, 44), *Gaffkya tetragena* (195, 196), *Hemophilus influenzae* (35), *E coli* (235), *B salmonicida* (61), *Mycobacterium leprae* (126), lactobacilli (12), *Klebsiella pneumoniae* (108), and *B abortus* (26)

Independent changes of characteristics have been observed in almost all of the more recent studies on dissociation and are illustrated by the following examples Humphries (108) demonstrated independent variation of changes in cell-morphology, instability in saline, inagglutinability in anti-S sera, and roughening of the colonies during dissociation in *K pneumoniae*, Osterman and Rettger (179) published data on independent changes of morphological and cultural characteristics during dissociation in the Friedlaender and coli-aerogenes groups, independent variation of colony morphology, cell morphology, production of somatic antigen, and pigment production have been reported in *S marcescens* (193, 194), and in *G tetragena* (195, 197) Gillespie and Rettger (77) working with *B megatherium*, and Mayer (151) with *Bacillus vermiforme*, have observed the absence of any definite relationship between cellular and colonial variation Pike (187) has reported the absence of any definite correlation between colonial morphology and the amount of capsular polysaccharide produced in variants of group A streptococci Sears and Schoolnik (212) showed that variation of fermentative abilities remained unaffected by S → R changes in *Shigella sonnei* Hershey and Bronfenbrenner (100) demonstrated the occurrence of variations in fermentative abilities in *E coli* without cultural or serological changes Independent changes of fermentation abilities and colonial morphology were also described for *Pseudomonas aeruginosa* (74), for *B anthracis* (173), and for *E coli* (178) Independent variation of colonial morphology, carbohydrate fermentation reactions, toxin production, and virulence have been reported (224) in hemolytic streptococci, changes in virulence without changes in colonial morphology have been also demonstrated in *Corynebacterium diphtheriae* (116), in streptococci (45, 93), in *Pasteurella pestis* (112), and in *M tuberculosis* (75), on the other hand, changes in colonial morphology without changes in virulence have been described (207) in avian tubercle bacilli Independent variation between polysaccharide antigens and virulence has been demonstrated for *Salmonella typhimurium* (145) Waaler (242) has observed numerous examples

of minor and large changes in the colonial morphology of dysentery bacilli without corresponding modification in the antigenic behavior (a variant having the S antigen could show a typical rough colony morphology, and an R variant with a rough morphology could change its morphology without any simultaneous alteration in the antigen), similar independent variation of antigenicity and colony morphology have been studied in *Noguchia granulosis* (95), hemolytic streptococci (91, 162), pneumococci (44), and *B abortus* (26, 31, 163), as well as in typhoid bacilli where independent changes of the "Vi" antigen and colony morphology have been described (62, 70) A change from "specific" antigens to group antigens without colonial type variation has been recorded in the *Shigella paradysenteriae* group (231), and in flagellated species the ability to form flagella has been demonstrated to vary independently from the S → R variation (*cf*, 60) Independent variation of toxigenicity and colonial morphology has been described in *Clostridium botulinum* (13) and in *Shigella dysenteriae* (21) Imšenecki (109) in his studies on independent variation in *Sarcina flava* has found identical ash content of R and S variants, equal resistance to high temperatures and no differences in their intensity of respiration, Planet and Bier (191) were unable to establish any correlation between phage-sensitive and phage-resistant types and colonial morphology in hemolytic streptococci, and finally, changes in sensitivity of pneumococci to sulfapyridine without changes in colonial morphology (155), as well as with changes in colonial morphology (154) have been recorded

As previously stressed by others, especially Humphries (108), Mayer (151) and Reed (194), this type of evidence fails to support all cyclogenic interpretations, and supplies indirect evidence for the mutation and selection concept, especially since instances of independent variation and the haphazard manner in which changes can occur in the presence of different selective environments are so frequent that it would be difficult to term them exceptions to the rule, as Hadley (87) has done Finally, this evidence against the ontogenic theory has been further strengthened by direct demonstrations of the manner in which various dissociation phenomena can be explained on the basis of mutation and selection (see below)

2 Evidence against the concepts of adaptation and "Dauermodifikationen" Convincing arguments against the adaptation concept have been listed by Mayer (151) These include the lack of direct correlation between specific environmental conditions and the type of change produced in populations submitted to them, the occurrence of widely different types under identical environmental conditions, the appearance of reversion under uniform conditions, and the fact that in dissociation completely different environmental agents can produce identical results

However, Mayer grants the rare occurrence of true modifications resembling the dissociation phenomenon in such cases as described by Paul (184) for Pneumococcus and Mallman (146) for Salmonella, where plating on specific solid media produced "pseudo-rough" colonies of Pneumococcus and "pseudo-smooth" colonies of Salmonella More recently, similar modifications to S have been

observed after growth of R forms of Salmonella in liquid media containing glucose supplements (20) and on solid media containing soluble starch (42) (see also p 99), also, pseudo-S forms of *Salmonella paratyphi* have been observed after growth on carbohydrate agar media (97) Non-hereditary variations in *B coli-mutabile* have been reported (255), such as mucus, capsule and alkali formation, which are induced by alkali engendered by other cells, and the production of pseudo-smooth colonies from rough tubercle bacilli when grown in the presence of egg-oil has been observed (228) Temporary changes in the colonial morphology of *Staphylococcus aureus*, observed after culturing in the presence of colchicine (244) might also be classified as modifications

Jollos' concept of "*Dauermodifikationen*" found its severest critics in Seiffert (214) and Lindegren (134) Principally, the same arguments as advanced against any type of adaptation applies to the "*Dauermodifikationen*" as well The haphazard manner in which dissociation may occur cannot be aligned with any interpretation which regards a temporary or semi-permanent change as the result of directing influences of the environment The aforementioned workers further stressed that the type of changes considered as adaptive can be equally well explained on the basis of mutation and selection During recent years the latter argument has found much support through data which have strengthened the views of those who regard the dissociation phenomenon merely as one manifestation of general bacterial variation (*cf*, footnote 8), rather than as "a special aspect or order of variability involving a special class of variants" (90) These newer data will be reviewed in the paragraphs to follow

IV DISSOCIATION AS PART OF GENERAL BACTERIAL VARIATION

As already noted, indirect experimental support for an understanding of dissociation in terms of spontaneous mutations and their subsequent selection has been contributed by studies which failed to agree with either of the alternative concepts proposed, but to which the mutation concept could be applied (134, 151, 194, 206, etc) The more direct support has come from work which has demonstrated the actual occurrence of spontaneous, undirected hereditary changes (mutations) in bacteria, and from studies which have elaborated the role of inherently controlled and environmentally modifiable mechanisms of population dynamics in the selection of mutant types Many of these newer data have had a definite effect on the understanding of bacterial variation in general, but since the recent advances in the field of general bacterial variation have been discussed by Luria (142a), brief reference will be made here only to those aspects which bear directly on the phenomenon of dissociation

1 Mutation The occurrence of mutations in bacteria, comparable in rates to those of higher organisms, has been established by the studies of Demerec (49, 50), Demerec and Fano (52), Luria and Delbrück (142), Tatum (232), Gray and Tatum (80), Roepke (202), Roepke, Libby and Small (203), Ryan et al (205), Lederberg (132), and Severens and Tanner (218) The mutations investigated by these workers involved changes in resistance and metabolic processes rather than the S, R, M changes typical of dissociation But Zelle (256,

257) working with *Salmonella typhimurium* contributed direct observations (by separating cells *in vitro* as they divided) of the spontaneous occurrence of one R cell during 148 divisions of S cells belonging to an "unstable" S type

Additional but somewhat more indirect support for the role of mutation in dissociation has also been derived from work which has demonstrated enhanced dissociation after treatment with x-rays, ultraviolet rays or radium emanation (24, 54, 76, 83, 84, 129, 133, 223, 237) all of which are known to increase the mutation rate of higher organisms [4] Also, acenaphthene, a chemical known to produce hereditary changes (polyploidy) in plants, has been found to produce mutations in certain phytopathogenic bacteria (6) The variants appearing in cultures submitted to these mutation-inducing agents differ quantitatively but not qualitatively from spontaneously occurring variants

The mutational origin of variants occurring during dissociation is further supported by the fact that dissociation does not occur in the absence of propagation (26, 92, 179, 211, 253), and by observations which reveal that the frequency of the appearance of variation is in proportion to the number of bacteria present (197, 214)

It is well known from research on higher organisms that mutational changes are undirected and spontaneous, generally occur at frequencies far below 1 per cent and are unaffected by the environment (except in the presence of such specific agents as radiations) Observations indicative of the same spontaneity of occurrence of variants in dissociation and data indicating the absence of any orderly direction of change under identical environmental conditions have already been referred to in the discussion of evidence against cyclogenic interpretations But it has remained for the valuable experimental work on mutations in bacteria by Luria and Delbrück (143), Demerec (49, 50), Ryan (205), and others, to supply the necessary direct evidence that bacterial mutations are really completely undirected and independent of the environment For example, it has been demonstrated that mutants resistant to penicillin or streptomycin occur independently of the presence of the antibiotics (49, 119), but are, of course, only detected in the presence of streptomycin or penicillin, when they attain unusual selective value There is valid reason to assume that changes in dissociation are similarly caused by the appearance of undirected spontaneous mutations, independent of the environment (196, 197), a point which becomes particularly convincing when it is realized that the customary studies on dissociation yield little information on the total types of mutations which may

[4] In this connection, it is interesting to note some hitherto unpublished observations on the similarity between ultraviolet radiation effects in *Brucella abortus* and selected unstable clones After irradiation of relatively stable S strains a considerable number of "ray like" colonies, displaying R and S sectors within one colony, have been observed (24) The same type of colonies occurs constantly in some recently isolated unstable S clones, they are thus indicative of increased mutation rates, which in one case were produced by irradiation of a strain with low mutation rate and are present, in the other case, by virtue of normally existing high mutation rates

occur, only the progeny of those mutants which have been able to establish themselves within the population can be detected, rather than the individual mutant (see p. 92).

This necessity of depending on the progeny rather than the recognition of the first mutated cell, also complicates the determination of the frequency (mutation rate) at which a variant cell may arise within an originally homogeneous population. This difficulty is inherent in the phenomenon of dissociation, where two or more types may coexist, but it has been overcome in the above mentioned studies on mutation to resistance. In the latter studies it has been possible to eliminate the original type (the non-resistant type) and a direct determination of the mutation rate has therefore been accomplished. The only comparable approach involving S → R variation is the already mentioned observation on individual cells as used by Zelle (256, 257). Somewhat idealized statistical methods which do permit the computation of mutation rates from the determination of variant types within a population subsequent to the first occurrence of the mutation have been presented by Delbrück (48). As already stated, the mutation rates obtained in all these studies, including the case of S → R variation, fall within the limits comparable to those of higher organisms.

Differential mutation rates have been clearly demonstrated in higher organisms. But differential frequency of appearance of bacterial variants in cultures exhibiting dissociation is not necessarily a reflection of differential mutation rates, as will be shown in one of the following sections. Nevertheless, it has actually been the analysis of this differential frequency of observed variants which has led to a better understanding of the dissociation phenomenon in terms of mutation and selection, because it has resulted in the recognition of certain factors which control the establishment of spontaneously arising mutants in populations, and which can be held responsible for many of the once contradictory problems that were encountered in the work on dissociation.

2. Factors controlling the establishment of mutants. a. Inherent factors. In 1937 Deskowitz (53) reported studies with unstable variants of *Salmonella typhimurium* which displayed stable ratios of variant types to original types. These ratios could be modified by changes in the environmental conditions, but were re-established upon return to the standard environment. Despite constant selection of the unstable type there was no detectable tendency to an increase or decrease in the ratio even after three years. Deskowitz stated that "unstable variation appears to be controlled by a definitely fixed inherent mechanism ... and that the final percentage of the two types in any medium may be a result of two factors: first, the actual rate with which stable cells split off from unstable cells—the genetic ratio, and second, the relative growth rate of the two types in any given medium." He concluded, "it seems probable that common bacterial dissociation and unstable variation are expressions of the same phenomenon, but differ only quantitatively." This statement has found subsequent support through observations on similar constant dissociation rate in hemolytic streptococci (221), through studies on differences in amount of

dissociation between two strains of *Staphylococcus aureus*, each isolated from a single cell (103), through reports on differences in dissociation potentials between single cells of *E coli* (235), and especially through the work on *Brucella abortus* (25, 26, 28)

In the studies on dissociation of *Brucella abortus* it has been possible to demonstrate the existence and nature of inherent factors which control the establishment of mutants within bacterial populations By studying the behavior of the direct offspring (clones) of isolated single cells under standard environmental conditions, it was established that the dissociation index, i e , the percentage of variant types in a 10-day-old broth culture originally inoculated with one type only, is constant for each clone and can differ significantly between clones Thus, members of one S clone when inoculated into standard broth would show approximately 3 per cent R and M (the latter has been often called Br in Brucella) types in their progeny after 10 days, those of another clone would show 80 per cent non-S types after the same period under the same environmental conditions

It was realized that these clearly inherent differences could be caused in two ways either the rate of appearance of variants (mutation rate) differed greatly between two such clones, or certain inherent factors controlled the differential establishment of mutants arising at equal rates within two clones with different dissociation indices It was possible to elucidate the importance of the last mentioned mechanism through the recognition of certain important features of bacterial population dynamics and through experiments in which the effect of environmental modifications upon clones with known dissociation indices were tested (For a detailed description see Braun, 26) The general conclusions from a large series of experimental studies were as follows

During population growth in liquid media the total number of cells increases steadily, whereas the number of viable cells (i e , the cells which have retained the ability to reproduce), regardless of the size of the inoculum, soon reaches a maximum which is retained as long as growth persists (see also Jordan and Jacobs (117), and the previous observations by Bail (11a), Wikullil (247) and Neufeld and Kuhn (172) on the so-called "M-concentration" in bacterial populations) Population pressure starts at the point of population growth at which the total number of cells becomes steadily greater than the number of viable cells This population pressure acts as a selection pressure, permitting the establishment of mutants with positive selection value, i e , in the presence of selection pressure any mutant with a higher growth rate or viability than members of the original population can establish itself rapidly at the expense of members of the original population, through more rapid propagation within the limits of the viable population The replacement of the original type by a mutant type with faster growth rate is easily comprehensible, but the importance of the differential between the total number of cells and the number of viable cells (viability) may need some explanation As an example, let it be assumed that for the original members of a population two out of every six arising cells

retain the ability to propagate, while two out of every three cells of the progeny of a spontaneously arising mutant propagate under the same environmental conditions Since the total viable population is limited to a maximum number per unit of the environment (while the total number of cells continues to increase!), the higher "viability" of the mutant type will suffice to replace the original type, even though the mutant type may actually have a slower growth rate (see p 94) Consequently, it was possible to demonstrate that the differential establishment of mutants in populations, expressed by differences in dissociation indices between clones of the S type for example, is due to the fact that the growth rate and the viability of the original members of a population determine the point at which population pressure starts, and the growth rate and the viability of mutants determine their chances to establish themselves within a population after population pressure has begun (*cf*, figure 9 in (26)) The inherent control of both growth rate and viability and their modification by certain environmental factors have been conclusively demonstrated in the work with *Brucella abortus* and have justified the conclusion that changes in growth rate or environmental modifications, can produce striking differences in dissociation indices *even at equal mutation rates*

The differential between total number of cells and number of viable cells, leading to population pressure and selection, was very great under the conditions maintained in the above experiments with *Brucella abortus* Although this high differential was fortunate from the standpoint of detecting the factors responsible for the establishment of mutants, it indicates that the media used were not ideal for optimum growth of Brucella More ideal media could be expected to produce a far less severe differential between the numbers of total and viable cells and consequently less or no population pressure, which in turn should result in a reduction or absence of dissociation Such conditions have been realized in recent studies on the growth requirements of Brucella (78), and it is noteworthy that no dissociation was observed under these conditions, a fact which supports the validity of the above cited results and interpretations These last observations may also serve as a demonstration for the contention that the establishment of mutants gets an increasingly greater chance with increasing intensity of conditions unfavorable to the original type (through reduction of viability of the original type leading to greater selection pressure and increased chances for the establishment of mutants with high viability under conditions adverse to the original type)

The more general applicability of the data obtained in the work with *Brucella abortus* is indicated by results obtained in an older investigation with two variants of *Staphylococcus aureus* (57) Differences in initial growth rates (checked by viable counts only) and optimum levels of viable population were observed, and these differences were found to account for the fluctuations noted between the percentage of each variant present at various times after the start of mixed cultures Earlier data indicative of similar mechanisms during the growth of pneumococcal variants can be found in the studies of Neufeld and Kuhn (172),

and also among the observations made by Mohr (164) for mixed cultures containing S and R variants of Salmonella, *Shigella dysenteriae*, and clostridia, respectively (see also p 90) [5]

b Environmental factors In the studies with *Brucella abortus* it could be ascertained that changes in environmental conditions which affect growth rates or viability, and thereby modify the factors controlling the establishment of mutants within populations, will alter dissociation indices Thus, dissociation indices can be modified by changes in the pH of the medium, changes in temperature, differences in available nutrients, or lowered oxidation-reduction potentials (26) Previously, such environmental effects had been noted by many others (e g , 37, 73, 130, 196, 241) and were usually vaguely expressed as influences of the medium upon a differential stability of types On the basis of the work with *Brucella abortus* it can be inferred that this apparently differential stability is merely the result of an environmentally induced modification of inherent factors which control the establishment of mutants in a population, and does not necessarily involve the initial appearance of mutants at differential rates

Undoubtedly, differential mutation rates may also play a role in the production of differential stabilities in various strains, e g , in the unstable variants of Salmonella (53), in certain unstable variants of Sarcina (76), and in the Brucella clones giving rise to sectorial colonies (see footnote 4), but the recognition of the role of inherent and environmental factors controlling the establishment of mutants suggests that a wide range of differential stabilities can be caused merely by the inherently determined and environmentally modifiable factors controlling population dynamics

The environmental conditions thus far mentioned can modify the degree to which a given mutant may establish itself within a population In addition there exist environmental conditions which can completely inhibit the establishment of certain mutants by suppressing their growth It has long been known that certain environmental conditions will favor one particular type only, for instance, it has been observed that the establishment *in vivo* of the smooth type (and in some species, the mucoid type) is usually favored (35, 46, 93, 99, 124, 127, 163, 181, 185, 257), and that the addition of antisera to cultures *in vitro* will prevent the establishment of the particular type from which the antibodies were produced (1, 46, 108, 146, 185) Such specifically selective environments have been utilized for the reversion of dissociative changes, e g , R populations which remained stable under ordinary conditions *in vitro* have been transformed into predominantly or totally S populations by animal passage or growth in the presence of R antiserum Similar selectivity has been claimed for the effects of

[5] At this point it should be remarked that, although the majority of experiments which have demonstrated the role of growth rates and other factors in the establishment of variants have been performed with liquid media, the general principles involved apply equally to the *dissociation phenomenon* observed on solid media In 1939, Shinn (220) contributed a stimulating discussion of the role of relative growth rates in the production of variational structures on solid media (wedge formation, secondary colonies), and his theoretical considerations are now amply supported by the more recent experimental work

temperature range (12, 22, 128, 165), phages (69, 124, 204), tissue extracts (174, 175), certain laboratory media (153, 233), atabrine (180), lithium chloride (86, 108), ferric chloride (111), iodine (239), sulfapyridine (238), soil (in the case of clover nodule bacteria) (176), products produced by one species affecting the dissociation of another species grown in association with it (2), pleuritic and ascitic fluids (114, 188, 189), and normal sera (1, 27, 28, 60, 108, 186, 198, 204, 221, 242, 243)

In the case of lithium chloride as a dissociation enhancing agent, the mechanism by which the selective action is produced may be postulated from studies with *Klebsiella pneumoniae* (105, 105a) which, as Sevag (216) has elaborated, indicated that Li inhibits the enzymes responsible for the synthesis of capsular polysaccharides It can be expected that in the presence of Li ions non-smooth organisms, i e , those which lack the inherent capacity of producing capsular polysaccharides, remain unaffected and attain an increased selection value, whereas the propagation and establishment of S-type organisms is inhibited through the interference by lithium with their normal enzymatic processes

Recent studies with *Brucella abortus* have also added to the information on the role of such selective environments in bacterial variation (27, 28) It was demonstrated that not only the addition of R or M antisera to beef-extract broth will suppress the establishment of R or M mutants, but that normal serum or plasma from man, cow, goat, hog, and rabbit are equally effective in preventing the establishment of R and M mutants Thus, addition of as little as 2 per cent of normal serum or plasma to broth cultures will prevent dissociation in an S population which under standard *in vitro* conditions (beef-extract broth only) would show 61 per cent dissociation after ten days Through the use of mixed S and R populations it was proved that after prolonged growth the R types do not increase in the presence of normal serum or plasma, whereas control cultures without serum or plasma showed a considerable increase of the percentage of rough over smooth types This inhibition of non-smooth types by factors present in normal serum is not due to the factors responsible for the bactericidal action of sera or plasma, since heating or filtration does not remove the inhibitive effect

Occasionally dissociation was observed in originally S Brucella cultures to which normal serum or plasma had been added, but the variants which establish themselves in these exceptional cultures are usually different from the types commonly observed (For further discussion of this point, see p 92)

Serum from the horse or chicken was found to be somewhat less effective in suppressing dissociation, and uncommon types of variants were noted in many cultures to which chicken serum had been added This difference in selectivity between chicken serum and that of the various other sera, as observed *in vitro* in Brucella, is paralleled by certain studies *in vivo* on the differential selectivity of host environments Buonomini (34) observed that animal passage of Sr forms of avian or SR types of human tubercle bacilli through hens tended to cause dissociation to the S form, while passage through guinea pigs tended to produce the R form, Ferreira (71) in his studies with avian tubercle bacilli similarly found that isolations from chickens always yielded S variants no matter what

type had been inoculated, while isolations from rabbits generally were of the same type as that inoculated Also, Jéline and Rosenblatt's observations (113) on organisms of the coli-typhoid group after their insertion (in filter-paper tubes) into the peritoneal cavity of various animals indicated selective changes in warm-blooded animals, but no selection in cold-blooded animals Among plant pathogens a similar observation has been made Nutman (176) found that the plant environment does not select variants of Rhizobium, while the soil environments exerts a strong selection in favor of certain variants The possible relationship of differential selective forces in various hosts to susceptibility of the host poses a challenging question for further studies

In addition to specifically selective factors already cited, it appears that the presence of sublethal concentrations of certain antibacterial agents, such as barium chloride (250, 251), phenol (61, 183), penicillin (209, 251, 252), sulfa-compounds (92, 154), and 2-methyl-1,4-naphthoquinone (41) also produce specifically selective environments, since it has been found that colony variants, especially small colony variants, with increased resistance to these agents, will become predominant under these conditions [6]

The inability of some colonial variants to grow on certain laboratory media which will support good growth of other colonial variants of the same species (229, 236, 246) provides an example of specifically selective environments which deserves special emphasis on account of its importance in the isolation and recognition of pathogens (see p 98)

Apart from the environmental effects which act by modifying factors governing population dynamics or selectively inhibit the establishment of particular mutants, a still different environmental effect upon dissociation has recently been described by Dickinson (54) She has demonstrated that the addition of certain organic acids to cultures of *Brucella bronchisepticus* will inhibit the mutational step *per se*, thus preventing the occurrence of dissociation in smooth cultures The necessary evidence that this constitutes a direct effect of the substrate on the mutational step was provided when it was observed that the presence of these particular dissociation-inhibiting substances did not at all affect the establishment of R types in mixed R and S cultures

c *"Association factors"* So far the role of inherent and environmental factors controlling the establishment of mutants in populations has been discussed The evidence for their importance in the phenomenon of dissociation can be considered well established Far less thoroughly investigated is the role of possible additional factors, which may be termed "association factors" These factors may play an important role in mixed populations where, in addition to competitive mechanisms produced by differential growth rates and viability,

[6] Incidentally, the available evidence tends to indicate that these small colony variants owe their lowered metabolism not merely to an environmentally induced interference or destruction of enzymes as some have claimed (154), and in which case they would have to be classified as modifications, they are in all probability true mutants with inherently lowered metabolism which permits their survival under conditions unfavorable for growth This assumption is based on the results which have been observed after their transfer to normal media (41)

the growth of one variant may decisively affect the growth of another variant During the early thirties such factors were vaguely postulated by some German workers Etinger-Tulczynska (68) reported on the antagonism between pneumococcal types *in vivo* and also *in vitro* Neufeld and Kuhn (172) continued these studies but added data on "direct antagonism" between S and R pneumococci of identical serological types, and Mohr (164) further extended these studies to observations on the establishment of variants in S and R mixtures of *Serratia marcescens*, *Salmonella* spp , *Shigella dysenteriae*, *Clostridium* spp , and *Brucella abortus*, respectively Whereas many results of these workers can now be interpreted as merely due to competition between variants with different selective values (differences in growth rate and viability), certain of the observations indicate the presence of additional unknown factors produced by one variant and inhibitory to the growth of another variant The above named investigators considered these factors as not metabolic in nature, since filtrates of cultures in which one variant had grown would not produce a similar effect upon subsequent growth of the other variant In the studies with Salmonella, Mohr observed the production of an equilibrium between the percentage of viable S and R types after prolonged growth, and this equilibrium remained the same regardless of the initial percentage of each variant inoculated at the start of the culture (These results are paralleled by some recent and unpublished experiments with *Brucella abortus* which also showed that after prolonged growth of cultures started with a mixture of S and R clones an equilibrium was reached between the percentages of viable S and R cells This equilibrium, expressed by the percentage of R types in the cultures after 10 days, was similar for a pair of clones regardless of the initial percentage of the two types, but differed in tests with different clones used for the original inoculum)

A more tangible case of "association factors" may be illustrated by citing the observations of Konst (123), who has shown that certain smooth cultures of *Mycobacterium paratuberculosis*, when grown in Long's synthetic medium, gradually render the medium more alkaline, whereas the rough type bacteria render the medium more acid At the same time Konst observed that the H-ion concentration has a modifying effect upon changes of populations from R → S Therefore, in mixed populations the pH change produced during the growth of one predominant type can be expected to affect the establishment of the second type Another "association factor" has been assumed by Zamenhof (254) in order to explain the delayed detection of mutant cells of *E coli* studied by him and others (182) He found the cause for this delay in the slow multiplication of the mutant and its early descendents in the mother culture, and ascribed this slowness to poisoning by a hypothetical inhibiting factor produced by the mother cells A good demonstration of "association factors" has been offered in studies related to the problem under discussion, namely in experiments on the distribution of color variants in ageing broth cultures of *Serratia marcescens* (32, 33) In these experiments it was observed that mixed populations change in a reproducible manner from cells producing predominantly dark red colonies to pink and white colony-forming cells and finally again to predominantly dark red cells

It could be shown that in fresh broth conditions prevailed which favored the establishment of dark red cells, but that this initial growth produced changes in the environment which in turn caused an increase in pale variants The nature of these environmental changes, produced by one component of the population, was indicated by observations which proved that an increase of pale variants could always be effected by adding autoclaved filtrate of old broth cultures or of autolyzed dark red cells

In the discussion of selective environments, it has been mentioned that in mixed populations consisting of two different species factors may exist which influence the dissociation of members of one species This is of some interest to the present discussion of "association factors" as well, the work which has been referred to was done by Alexander and Cacchi (2) who observed that after growth in association with *Lactobacillus bulgaricus* there was a marked tendency of originally S-type typhoid-paratyphoid organisms to produce R colonies (pH effect?) Similar results have also been reported for mixed cultures of nodule bacteria and *B mycoides*, and even for mixed cultures of bacteria and amebae (122)

3 Phenomena involving the interaction of inherent and environmental factors The elucidation of the role of inherent and environmental factors in controlling the establishment of mutant types in bacterial populations has contributed to a better understanding of a number of problems encountered in the phenomenon of dissociation

a Dissociation and mutation patterns One problem which now can be better understood, concerns the "dissociation pattern" It has not only been noted that a strain may produce different variant types depending on whether dissociation takes place *in vivo* or *in vitro* (185, 186), but it has also been observed that different strains or clones of one type may yield different variants under constant environmental conditions (26, 103, 165, 240) The latter must be attributed to differential mutation patterns inherent in the organisms, i e , an inherent tendency of a given mutation to occur more frequently within one clone than in another In the case of different dissociation patterns under different environmental conditions, however, the type and rate of mutation may be alike, whereas the selective value of arising mutants, and consequently their establishment, differs considerably within different environments As an example of different dissociation patterns, the uncommon variants observed after addition of normal serum to *Brucella abortus* cultures may be mentioned (27) As previously noted, populations from S clones, which after prolonged growth in standard broth showed a high percentage of R and M mutants, remained smooth in the presence of normal serum, in a very small percentage of cultures, however, dissociation was occasionally observed even in the presence of serum The mutant types in these exceptional cultures were strikingly dissimilar from those observed under standard *in vitro* conditions It was assumed that these uncommon variants represent the offspring of mutants which normally may arise but which cannot establish themselves when they are in competition with arising R and M mutants Only when the establishment of the

latter types is prevented through the already discussed factors present in normal serum, will the uncommon types attain a sufficient selection value to permit their establishment Since, even in the presence of identical mutation patterns, the establishment of any arising mutant must thus depend upon the selective influences of the environment, as well as the selective value of other competitive mutants present, it is obvious that only a small range of potential mutation patterns can be observed under any given environmental condition For the detection of the total potential mutation pattern, it would be necessary to submit a strain to a variety of different environments, the total of which would permit the establishment of every arising mutant

b Reverse mutations In many studies on dissociation it has been observed that it is difficult to reverse the trend of dissociation from R → S *in vitro* This difficulty is easily explained when it is realized that such a reversal would necessitate the establishment of smooth mutants, with comparatively low selective value under *in vitro* conditions, within a population of rough types which have been able to establish themselves within an originally smooth population through their higher selective value (27) Thus, a smooth mutant may arise in a rough population, but it will have little chance to establish itself because it is competing with members of a population which have already a higher viability or growth rate than the smooth mutant Under environmental conditions which are identical with those which have permitted the establishment of one mutant a true reversal to a population consisting of the original type can therefore not be expected If, however, environmental conditions are changed so that the original type (e g , the S) attains a higher selective value than the mutant (e g , the R), then a true reversion in the population type (e g , R → S) can manifest itself Such is the case in the presence of serum, and under *in vivo* conditions In addition, an *apparent reversal* can be found under constant environmental conditions, as it has been observed with certain rough clones of *Brucella abortus* which showed a large percentage of smooth types after prolonged growth in broth (26) However, despite their morphological and antigenic similarity to the original S type (from which the R type used had originated), these particular smooth types, when more thoroughly tested, proved to have a faster growth rate than both the original S *and* the R type from which they arose, thus permitting their establishment A process which superficially looked like a true reversible change S ⇄ R, under constant environmental conditions, therefore actually represented a change from S → R → S_1, in which S and S_1 indicates types which are identical in all characteristics except those which determine their selective value under constant environmental conditions The older observations of Spicer (224), and Mellon *et al* (162) on R → S reversions in hemolytic streptococci, where distinct differences between the "reverted S" and the "normal S" were observed, may now be similarly explained The same applies to other examples of differences between "original" and "reverted" S types cited by Hadley (87) (It may be noted that some investigators (e g , Winslow, 248) have argued that the occurrence of reverse changes in bacteria indicates that bacterial variation is not comparable to mutation processes in

higher organisms since reverse mutations do not occur in plants and animals This argument has become completely invalid because the occurrence of reverse mutations has by now been amply demonstrated in higher organisms)

c *The production of apparent cycles* Undoubtedly, the problem of apparently orderly successive changes or "cycles" has been of major importance in studies on the nature of bacterial dissociation, especially since it has led to the construction of "cyclogenic" theories which refuted the validity of interpretations based upon mutation and selection It is, therefore, significant that recent information has supplied evidence which illustrates how competition between

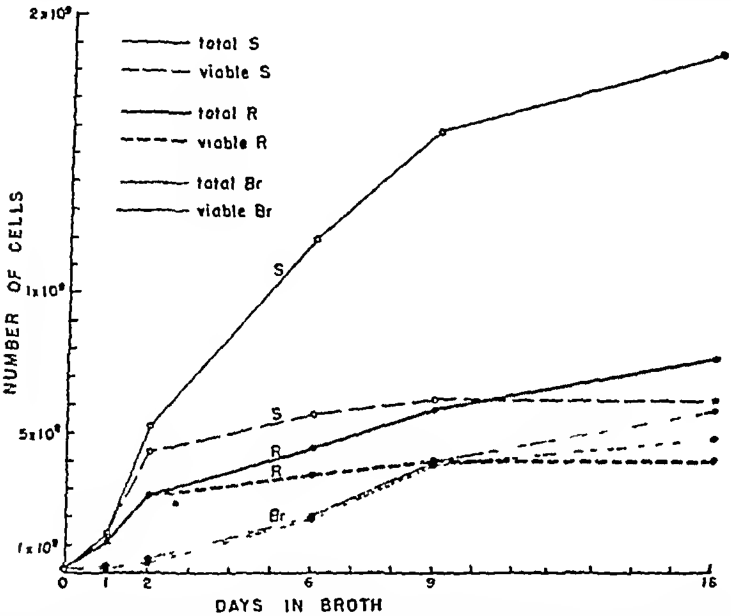

FIGURE 1 Total number of cells and number of viable cells in broth cultures of S, R, and Br (= M) types of *Brucella abortus* on various days after starting cultures (From the Journal of Bacteriology (30))

spontaneously arising mutants with different selective values can produce the appearance of successive orderly changes (27, 30) Again, the crucial fact is that of all spontaneously arising variants only those can establish themselves which have a higher selective value (growth rate or viability) than the original members of the population Thus, several variants which arose from one clone of *Brucella abortus* were analyzed, and it was found that the S type had the fastest growth rate (increase in *total number of cells*), R types had a slower growth rate and M types a still slower growth rate, but M types had the highest viability (ratio of total number of cells to number of viable cells per milliliter of broth), R types a slightly lower viability, and S types the lowest viability (see Figure 1) Simple calculations based upon these observations proved that as soon as these R and M types arise within a smooth population their higher

viability, despite their slower growth rates, will suffice to permit their establishment at the cost of S types among the limited viable population The R types will become predominant first and they will eventually be replaced by the M types with the even higher viability but much slower growth rate Thus, merely by spontaneous appearance of mutants differing in growth rates and viability an apparently successive change from S → R → M can be produced under constant environmental conditions

Changes in environmental conditions which alter the selective value of any such mutants can be expected to alter the order of their establishment, and this has been observed in the previously cited older studies which were concerned with alterations or even reversals of the apparently cyclic changes during dissociation Furthermore, it would be impossible to observe such successive establishment of mutants with different selective values, under environmental conditions which selectively suppress the establishment of certain mutants This can be recognized as the phenomenon which has been called "stabilization of the culture phase" by Hadley (86, 87) [7]

4 Causes for the absence and presence of linked variation One of the typical manifestations of the phenomena grouped under the label of dissociation is the frequent correlation of changes in several characteristics The occurrence of this linked character variation, and the many exceptions illustrating the occurrence of non-linked changes have been discussed In assessing their probable causes two different mechanisms must be considered (29)

First, a great number of constantly linked character changes must be attributed to one basic change only, e g , loss of capsular polysaccharide, which conditions the expression of other characters dependent thereon (60)

Second, in cases where a set of characters may undergo linked as well as non-linked variation, a single mutation at different levels of gene-controlled processes can be held responsible This type of interpretation has been successfully applied to experimental results observed in genetic studies on higher organisms and has been suggested for an explanation of metabolic changes among virus-resistant mutants of *E coli* (5) The manner in which this may be applied to changes observed during dissociation is illustrated in Figure 2 A mutation at level A, affecting process *a*, and thereby all subsequent processes, will cause a change in colony morphology, cell morphology, stability in saline, and virulence, another mutation (B) will affect process *b* only and thus cause a change in virulence without changing colony morphology, cell morphology, and stability in

[7] The demonstration of the mechanism by which apparently cyclic changes may be produced in the presence of completely undirected, spontaneous changes adds decisively to the arguments which have been advanced for a rejection of life cycle interpretations in the sense which has been advocated by Hadley The concept of life-cycles, as used by Hadley, was supposed to denote the existence of some directed potential of an organism to pass from one "cyclo-stage" to a more "advanced" one, provided the environmental conditions permitted its expression In the strictest sense of terminology, however, the term life cycle could possibly be applied even to the manifestations of the above described processes, although the connotation of the term would differ fundamentally from that advanced in Hadley's writings (*cf* , 249)

saline, still another mutation may occur at level C, affecting process c, and thus cause changes in colony morphology, cell morphology, and stability in saline without altering virulence, and so on.

Several examples of experimental work supporting such an interpretation have been cited during the discussion of independent variation between several characteristics (p 81), another good example is supplied by the work of Shaffer *et al* (219) They studied two S strains (Griffith's terminology) of Pneumococcus, which were both highly virulent for mice, but differed sharply in their virulence for rabbits The two strains exhibited differences in time of growth at which a reduction of the capsule occurred, and correlated with this was a

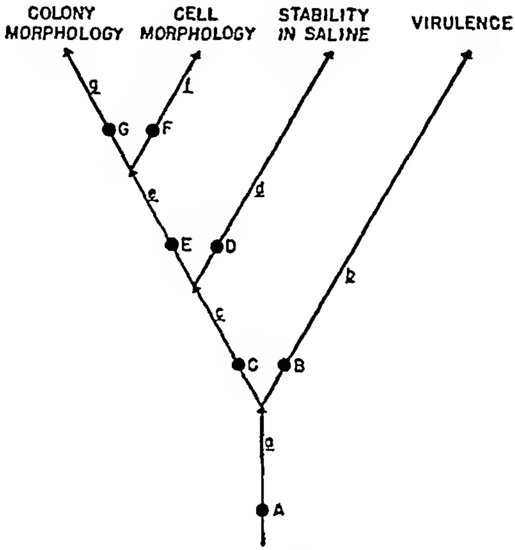

FIGURE 2 Diagram illustrating a mechanism by which the occurrence of linked and non-linked variation of characteristics may be caused (The order of characteristics in the above diagram has been arbitrarily chosen, for further explanation see text)

shift of the range of acid-agglutination, susceptibility to clumping in anti-R serum and ingestion by leucocytes R variants which were obtained from these two S strains were distinguishable from one another in morphological and growth properties Upon reversion to S the same differences in virulence recurred The investigators suggested that the factors upon which the difference in virulence between the two strains depends are the product of stable physiological processes of the bacterial cell which are retained during the transformation S → R → S, which, under the scheme outlined in Figure 2, may now be interpreted as a mutation that affected a shift in colony morphology and certain antigenic properties without affecting other processes determining differences in virulence and growth rates, and a subsequent reverse mutation at the same level

In addition, Luria's recent work (142) has indicated still another mechanism by which non-independent mutations may arise in bacteria, namely an influence of one mutation on the rate of occurrence of another mutation His data, obtained in studies on mutations affecting resistance to bacteriophages, have led him to the belief that the wild type allele of one gene may act as a suppressor for manifestation of the effects of the mutant allele of another gene

V THE RELATION OF DISSOCIATION TO SOME BASIC AND APPLIED PROBLEMS

Even in the absence of exact knowledge regarding the nature of dissociation, the possible relation of this phenomenon to problems of taxonomy, epidemic waves, immunology, and the course of the infective process, has been extensively discussed (87) These discussions have retained their general validity despite changes in the interpretation of the nature of dissociation

1 Taxonomy Hadley (87, 89) and others (e g , 60, 192) have argued that the classical method of classification of bacteria is quite inadequate, since it is based upon a single form ("culture phase" in Hadley's terminology) which is regarded as typical for the entire species, while, according to Hadley, a species is typified by the "summation of all its phases together with their individual attributes" In the light of more recent contributions this objection may be restated as follows current classification is based upon the characteristics of one mutant type without regard to forms with altered characteristics which may occur within the viable mutational range of each species The exact classification of bacteria is far more difficult than that of higher organisms in which sexual mechanisms permit the detection of species barriers through cross sterility In the absence of such detectable barriers a representative mutant chosen as a species type can serve as a helpful tool for classification, but it must be recognized that mutational ranges of species may overlap, resulting in the often confusing observations that the progeny of one species may display characterists typical of another species, or even species belonging to different genera (Mellon's (160) "pleobiotic stages") For example, some R mutants of Pneumococcus are indistinguishable from certain strains of *Streptococcus viridans* (185) *Alcaligenes radiobacter* and *Phytomonas tumefaciens* show no serological similarity when their M types are tested, but antigenic identity was found when the S types are tested (40), the antigenic gulf usually existing between the hemolytic streptococci and the non-hemolytic diphtheroids has been bridged by variants of the former, making for an antigenic continuity between what have been traditionally regarded as distinct genera (160, 162), and the usual definition of the Friedländer group does not necessarily exclude organisms of the coli-aerogenes group when they are represented by the M type (179) It has also been claimed (125) that both hemolytic and green streptococci can be "dissociated *in vivo*" from mice which have been inoculated with pure cultures of pneumococci Furthermore, it has been noted that related species which have been considered as distinctly different from each other are nothing else than stabilized variants of the same species (74, 104)

An improved method of classification would involve the description of the

total potential mutational range of each bacterial species (*cf*, 222), however, such a method would be far too cumbersome for practical purposes The more practical approach, utilizing mutants which are predominant within the "natural" habitat as species type, is usually striven for in present taxonomical practice But this method may lead to erroneous results if it is failed to realize that the so-called normal type represents merely one mutant which has been able to establish itself under specifically selective conditions (27), and that any change in environmental conditions may lead to the establishment of other mutants It is, therefore, important to test bacteria immediately after primary isolation and to choose environmental conditions for their growth which resemble those of the natural environment The importance of environmental conditions represented by laboratory media used for the isolation of organisms has been stressed by Stuart *et al* (229) after their observations that some colonial variants of *Shigella sonnei* and cultures of anaerogenic paracolon bacilli are unable to grow on certain media commonly employed for the culturing of these species They pointed out that if variants which fail to grow on media used to isolate enteric organisms occur in nature and are pathogenic, sporadic cases and epidemics could occur in which ordinary technique would fail to ascertain the etiological agent It has also been realized, and put into practice, that for the classification of types represented by mutants lacking important characteristics (such as type specificity or fermentative abilities), e g , the R type of Salmonella or an M type of Streptococcus, it is necessary to study the corresponding S type within the mutational range of the species (63, 147), this is accomplished by growing the R type in an environment which will favor the establishment of S mutants Similarly, Morton and Shoemaker (169) have reported the identification of small colony variants as *Neisseria gonorrhoeae* only after their change into a large colony type, and Mellon and Beinhauer (161) have reported the recovery, from a patient with anomalous tuberculosis, of an actinomycotic organism which, upon dissociation *in vitro*, produced organisms which culturally and in other ways were indistinguishable from a variant of the virulent S form of the Koch bacillus The improved recognition of factors involved in the establishment of mutants should aid considerably in such procedures

The phenomenon of dissociation thus plays an important role in the taxonomy of bacteria The similarity of its manifestation throughout various bacterial species suggests that the mutational steps involved affect similar processes, the characteristics which determine species barriers, however, must have reached stabilities which cannot be affected by ordinary mutations and lead to viable progeny An additional point deserves reiteration here, namely the previously discussed feasibility of having one mutational step affect several characteristics Sudden changes involving several characteristics have led to types which are often difficult to classify and their position in the framework of established taxonomy has puzzled many bacteriologists who believed that one gene controls merely one character and one mutation can affect only one characteristic The fallacy of this belief has been established in higher organisms and there is no reason to assume that genetic mechanisms in bacteria differ in this respect In

concluding the discussion of the relation of dissociation to taxonomy, it may be stated that a clear distinction should be made between the inherent potentials of a bacterial cell and their manifestations For example, even though the rough type of Salmonella fails to manifest any type specificity, it must contain the inherent potential for it, because, as recent results (42) indicate (see p 83), as soon as R is modified to S by the presence of sufficient polysaccharide (presumably the polysaccharide synthesis and capsule formation have been disrupted by the S → R mutation) the genetically R type will show distinct type specificity Similarly, R types of Pneumococcus will remanifest the same type specificity as their ancestors when they revert to S (Griffith's terminology), unless this inherent potential is permanently altered through the presence of specific desoxyribonucleic acid, as demonstrated in the stimulating studies of Avery and co-workers (11, 110, 152)

2 Epidemic waves Hadley (87, 90) and others (e g, 81, 102, 141) have suggested that there may be a relationship between dissociative changes, accompanied by changes in virulence, and the problem of epidemic waves While this subject still remains somewhat speculative, additional consideration may be given to it on the basis of recent information The demonstration of naturally occurring blood factors, which selectively restrict *Brucella abortus* populations to the S type, may be of some significance in this connection, if a similar activity of *in vivo* components can be demonstrated for other bacteria It may be assumed that in the presence of these factors any invading bacterial population is restricted to the usually virulent S or M type (depending on the species, see p 78) With the production of antibodies the selective environmental conditions will change and spontaneously arising avirulent mutants, such as R mutants, may attain a survival value Consequently, if a host survives the initial invasion by the virulent parasite, non-S or M mutants may establish themselves and thus produce a carrier

Such speculations are supported by a number of observations for which the following may serve as examples Marconi (149) isolated *M tuberculosis* from purulent bone abcesses of six patients and observed a tendency to dissociation from S to R as the processes tended to become chronic, Pisu (190) isolated 38 strains of *Shigella* from a single source of epidemiologically related cases and noted that S forms were isolated in the beginning of the epidemic, SR forms as the epidemic waned, and R forms towards the end of the epidemic, R types have been isolated from chronic carriers of typhoid bacilli (81) and *Erysipelothrix rhusiopathiae* (96, 210), and Griffith (82) has published observations on the isolation of R variants of avian tubercle bacilli from the udder of a goat 8½ years after the original inoculation with S types In an investigation of streptococcal infections, the isolation of virulent mucoid types from acute infections and isolation of avirulent smooth types from subacute or chronic infections have been reported (92) Another interesting observation has been described in a case of gonococcal infection, where dissociated forms were isolated from a chronic patient, but when the organisms entered a new medium (the patient's wife) only the common virulent types were isolated (79) Also, Almaden (3)

has reported the isolation of virulent M types during acute pneumococcal and gonococcal infections, and the isolation of S types during recovery The contention that such changes of the bacterial population are intimately associated with changes in the selectivity of the host environment is upheld by further observations *in vitro* on the effect of sera on dissociation in *Brucella abortus*, (27) whereas normal sera contain factors suppressing the establishment of non-S mutants, sera from vaccinated animals will permit the establishment of R and M mutants Presumably, under such conditions the suppression of S types due to the presence of S agglutinins is stronger than the suppression of R and M variants by factors normally present in serum Similarly, in work with other bacterial species, the establishment of avirulent variants in immune sera (10, 111) and in immune hosts (67, 112) has been described

Once the R type (or other non-virulent variants) have established themselves in disease carriers, a subsequent decrease in S antibodies may again alter the selective environment by permitting the natural anti-R factors to exert themselves, and virulent S mutants may then re-establish themselves. Another possibility, suggested by certain pathological findings, is that S types may be removed from the selective environment (e g , through fibrinous encapsulations) and a population change may occur in the local absence of selective conditions A release of members of this changed population into areas subjected to the selective factors would then in turn re-establish S mutants Further research on possible changes of selective *in vivo* factors during acute infections and in chronic stages will be required for more exact information on this possible relationship between epidemic waves and bacterial variation

3 Immunology The differential immunogenic potency of different variants has been widely recognized and has also been discussed by Hadley (87, 90) The utilization of non-virulent variants in the practice of immunization is also well established (123) Further work has substantiated the *importance* of selecting appropriate variants for the production of vaccines (e g , 4, 14, 72, 94, 131, 234) and protective sera (136, 144), and in a number of instances the separation of fractions with immunizing value from particular types, like the S type of Pneumococcus, has been accomplished (58, 59, 60, 156) Whereas the S or M type has usually been utilized for the production of efficient vaccines, e g , the S type in the control of brucellosis, the possible immunizing value of other variants, i e , the M type in the case of Brucella (107) and the R type in the case of hemolytic streptococci (224) and *Erysipelothrix rhusiopathiae* (210), has been suggested (However, in the case of the hemolytic streptococci, it was later established that the protection produced by the R type is non-type-specific and appears to be associated with the increased mobilization of monocytes in the peritoneal cavity rather than with any specific immune mechanism (23)) In view of the importance of the correct variant type for vaccination purposes, the control of dissociation in the production of vaccine has attained considerable practical importance Improved recognition of factors controlling dissociation and the use of selective environments favoring the establishment of the desired type can be expected to aid in avoiding difficulties introduced by dissociation

during the production of vaccines and the storage of live vaccines Moreover, the demonstrated feasibility of selection of clones with superior characteristics from the heterogeneous populations represented by morphologically identical types (26) suggests that methods of selection for organisms with desirable inherent tendencies will lead to further improvement of vaccines and, thus, to improved disease control In other words, improved immunological procedures do not merely involve the selection of one type only, like the S type, but additional isolation of particular clones with superior characteristics from the many mutants grouped within the range of S types

VI GENERAL CONCLUSIONS AND SOME IMPLICATIONS FROM RECENT RESULTS UPON FUTURE WORK

On the basis of the above reviewed evidence, the phenomenon of dissociation appears to resolve itself into merely another manifestation of causally related mechanisms of discontinuous[8] bacterial variation which involve the spontaneous occurrence of undirected variants (mutants) and their subsequent establishment within a population under the control of inherent and environmental factors governing population dynamics[9] Changes which occur during so-called dissociation do have certain distinctive characteristics, these include frequently linked character variation and the occurrence of numerous mutants which, on the basis of colonial morphology or antigenicity, can usually be classified into several general groups or types of mutants, such as S mutants, R mutants, M mutants and I mutants[10] But it has been demonstrated that these characteristic manifestations, as well as other problems once considered as unique for the variational aspects grouped under the term dissociation (e g , the production of apparent cycles), can all be explained satisfactorily on the basis of mutation and selection

The term dissociation as an indicator of a particular mode of bacterial variation can, therefore, be considered superfluous, and may actually be misleading if used to denote a change of characteristics *per se*, the continued use of the term will only be justified if applied to population phenomena, i e , the spontaneous change of one or more members of a bacterial population and their subsequent establishment

This review has concerned itself purposely with only a small range in the larger spectrum of bacterial variation, but the results which have been accumulated within this range support the conclusion that the general nature of discontinuous bacterial variation, including the phenomenon termed dissociation, manifests no fundamental differences from the more thoroughly investigated

[8] The term "discontinuous" is here used in the sense advocated by Dubos (60) to distinguish one group of variational aspects from others mentioned in footnote 9

[9] Apparently causally different from these mechanisms of bacterial variation are *a*, the variational aspects termed enzymatic adaptation, which may occur in the absence of propagation and, *b*, many of the cell pleomorphisms which can be attributed to purely physiological modifications

[10] Within each of these groups many different mutants with various characteristics and varying degrees of "smoothness," "roughness," etc , may be found

processes of genetics in higher organisms, except for the absence of a clearly developed sexual mechanism of reproduction It is the absence of the latter, *plus the far higher* reproductive rate of bacteria, and the intense population and selection pressure existing in bacterial populations, which may be held responsible for the apparently greater instability of bacteria, their seemingly specific adaptations to natural environments, and their ability to adapt themselves rapidly to altered environmental conditions (27, 30a, 134) In other words, the apparent difference between stabilities of higher organisms and bacteria, which once was used as an argument against the application of mutation concepts to problems of bacterial variation, is evidently not due to any greater instability of the determinants of inherent properties in bacteria, but to the greater chance which exists for microörganisms with changed inherent properties to establish themselves under the conditions of intense population and selection pressure in the absence of masking sexual mechanisms It has been proved that, even at mutation rates comparable to those of higher organisms, changes in bacterial populations will proceed rapidly if the mutants possess sufficient selection value to establish themselves within a population with lesser selection value under given environmental conditions

So far as the actual determinants of inherent properties in bacteria are concerned, the evidence for an existence of cytological units comparable to the nuclei and especially intranuclear chromosomal structures of higher plants and animals is still too scant to justify concrete analogies between the site and structure of genetically determinative units in the bacterial cell and the organized nucleoprotein compounds (chromosomes) of higher organisms In the absence of sufficient information on this subject, it is also of little value to speculate on the actual mechanism of inherent changes involved, whether it may be in the nature of transgenation (134, 135), a loss variation (177, 194), or a rearrangement of nucleoproteins However, the enhanced interest of many geneticists in the problems of bacterial variation, coupled with new cytological observations (e g , 199) and newer biochemical approaches similar to those used by Avery *et al* (11, 152) may soon lead to more information on this subject (It may be added that recent demonstrations indicative of the role of extranuclear hereditary (autocatalytic) units in higher organisms (*cf* , 225) has somewhat removed the nuclear elements from the overall dominant position which they had attained in the field of genetics, and these advances may well be of some significance to genetical problems of organisms in which an organized nucleus is usually difficult to recognize)

Some of the implications from recent results upon future work in the field encompassing the phenomenon of dissociation have already been indicated in the discussion of taxonomic, epidemiological, and immunological problems and their relation to dissociation In addition, there exist promising indications that the better understanding of the variational mechanisms underlying dissociative phenomena, combined with feasible biochemical investigations on bacterial variants (5, 17, 80, 101, 152, 217, 226) will produce future basic information on the nature and transformation of hereditary properties which will be of *interest*

both to bacteriologists and to geneticists Certain interesting correlations between phenomena of bacterial variation and some general evolutionary as well as morphogenic considerations have already been discussed elsewhere (30a) and shall be mentioned here only in passing

But there remains one further practical implication arising from recent studies on dissociation which deserves brief discussion here, namely the possible relation of certain above reviewed results to new methods of controlling infectious processes Hadley (87) has discussed this possibility to some extent and in his terminology he stated it as "a new aim and method for combating infectious diseases by controlling the culture phase of the infecting agent", which now may be restated as attempts to affect the establishment of non-virulent mutants in hosts which have been invaded by virulent bacteria Mellon (159) believed to have contributed a direct experimental approach to this problem in his apparently effective attempts to cause transformation *in vivo* of populations of virulent hemolytic streptococci into populations with diminished virulence through administration of antisera prepared from anomalous variants The newer information on the existence of selective factors in normal sera, preventing the establishment of non-S types (27), supported by the additional information on the establishment of variants in immunized hosts (67, 112), as well as in carriers (81, 82, 92, 210), and the interesting observations on the establishment of avirulent variants in sulfonamide treated animals (154), now adds to the potential feasibility of such an approach Accordingly, in one special study attempts are now being made to isolate the blood factor responsible for the suppression *in vivo* of R and M variants of *Brucella abortus*[11] Upon isolation of this factor, its inactivation will be tried *in vivo*, thereby attempting to create a change in the selectivity of the host environment comparable to the conditions existing *in vitro* where, in the absence of the blood factors, the establishment of avirulent types is favored In this connection some recent results of Watson (245) may be of interest He has been able to isolate an anthraxcidal substance from the blood of animals possessing different degrees of normal resistance to anthrax infections The substance proved to be of histone-like nature, with an unusually high lysine content, and was present in greater quantities in animals with high natural immunity The substance is inactivated by thymonucleic acid but not by ribonucleic acid, a point which may be of general biological significance But of greatest significance to the subject under discussion is the fact that the virulent anthrax type is a rough type and that, therefore, the substance isolated by Watson is a normally occurring compound affecting a rough type, which may possibly be identical with or similar to the normally occurring serum factors against R types which have been specifically investigated in Brucella

In conclusion, attention may be called to the use to which smooth and rough organisms may be put in assaying the antibacterial activity of chemical entities in penicillin samples (208) It has been shown that when organisms change

[11] This blood factor has just been found in the gamma globulin fraction of normal plasma.

from the smooth to the rough type the response to one of the penicillin entities may increase by almost 100 per cent, whereas the response to other penicillins may remain constant This represents an example of the practical application of S and R mutants to testing procedures in which they may assist in the identification, use and development of antibiotics, and thereby aid the advancement of a currently highly import field of therapy At the same time this demonstration involves the application of variational aspects of resistance (which have lately received so much attention in genetic studies on bacteria), as well as it involves those variational aspects classified under the term dissociation, therefore it may serve as a final example of the inadequacy of divorcing the so-called phenomenon of dissociation from other manifestations of general bacterial variation

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USE OF MOVING AVERAGES AND INTERPOLATION TO ESTIMATE MEDIAN-EFFECTIVE DOSE

I. FUNDAMENTAL FORMULAS, ESTIMATION OF ERROR, AND RELATION TO OTHER METHODS[1]

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Estimation of the potency of a given reagent by means of its action upon living matter is the purpose of biologic assay. Test designs and methods of evaluation of results have been developed extensively through collaboration of biologists

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and statisticians Methods of estimation of median-effective dose[2] (M) have been a critical feature of many systems of *quantal* assay, where the response of test-individuals is *all or none* The present article describes a method of moving-average interpolation to estimate M from the assay data Section headings and table of contents may serve as a guide to information essential to its actual use or to an understanding of its fundamental character and relation to other methods Fine print has been used where discussions serve more as amplification or aid to a careful study than as development of the main themes On account of the relative simplicity of assumptions and calculations involved, the present method may be preferred unless the use of some other method in a given situation can be justified by improved precision or by permitted technical economies It is in this sense that moving-average interpolation is suggested as a *basic method*, not in the sense that it is more elegant aside from this dual simplicity Attention is directed also to judgment of possible influences of choice of test-plans upon precision

INTRODUCTION

In biologic assay based on quantal, *all or none*, response it has been found widely advantageous to use a sequence of doses (D_i) in geometric progression, for $i = 1, \quad , h$, each administered to a given number n_i of subjects (such as animals or eggs), and it seems preferable to plan to have the same number of subjects in each case, $n_i = n$ In each case the number r_i that respond critically may be tabulated, against the corresponding dose D_i in ascending order of magnitude In any case the n_i subjects and results of administration of the dose D_i are considered as a sample from a hypothetical universe of all possible individuals of the sort (respectively subjects or results) presumably obtainable by

[2] The writer's attention has been called to the unfortunate fact that M has been widely used with a different meaning elsewhere in the literature on bioassay, as by Gaddum (22) to represent the logarithm of the ratio of two potencies To facilitate the present discussion, in describing the logistic relation, we here employ M, D, p, and G respectively where Thompson and Maltaner (7) and also Kent *et al* (9) used K, x, y, and h or $1/n$, and where von Krogh (2) used k, x, y, and $1/n$

It is much more convenient here to use M to denote the median-effective dose than alternative notations such as ED_{50} Moreover, this is in accord with the notation employed in the chapter on biologic assay in the Standard Methods (5) of this laboratory which also employs confidence ranges for the median M with the same notation used in their original development (36) in a form yielding certain exact probability statements, *specific confidence* that any given percentile of essentially any universe lies below the l-th observation in ascending order from a random sample of aggregate number n The article has been discussed in a review by C C Craig (39a) Use of any other symbol here instead of M would seem to place an unwarranted burden on cross-reference Furthermore, M for the median occurs in many statistical texts, e g, Rietz (29), Camp (40a), and Rider (28), others employ more cumbersome symbols, such as Mi, Md, Med, or Q_2

It is suggested that an *italic* or *script* L or λ might be used for the logarithm of the ratio of two potencies instead of Gaddum's M, or a *script* M be used for the median or median-effective dose instead of the present M, if economy demands single-lettered representation of each in the same discussion The second alternative seems less attractive since the symbols do not suggest their meaning

more extensive experience. Such a universe is conveniently called the sampled *population*, usually considered as having an infinite aggregate number. Let $p_i \equiv r_i/n_i$, and $\bar{p}_i$ be the hypothetically unknown *true* probability of critical response to the dose D_i for the sampled population. Then, the usual immediate objective in such a bioassay is to estimate the *median-effective* dose M, defined as a dose such that, if $D_i = M$, then $\bar{p}_i = 0.5$ (i.e., M is the dose that should be *expected* to yield a 50-per-cent response, for example, the LD_{50}).[2]

For convenience in discussion, let the hypothetical curve of points (log D, $\bar{p}$) be called the *fundamental curve*, and consider its hypothetical graph to have log D as abscissa and $\bar{p}$, the corresponding *true* probability of critical response, as ordinate. If some function of $\bar{p}$ is used instead as ordinate, say $T_{(\bar{p})}$, let the corresponding points and curve be regarded as *transformed* from the fundamental coordinate system (log D, $\bar{p}$), consider experimental points (log D_i, p_i) as likewise *transformed* to points (log D_i, $T_{(p_i)}$).

The usual purpose of such a transformation is to straighten the fundamental curve so that a straight line may be fitted to the transformed points and M be estimated by M' from the point (log M', $T_{(0.5)}$) on the fitted line, corresponding to $p = 0.5$. The possibility of a gain in power of estimation by such methods is obvious. However, there is imminent danger that tendencies toward biased or erratic estimates may be induced by mistaken assumptions about the form of the fundamental curve or by the technics used for curve fitting. Even for no other purpose than to serve as a basis of comparison, it would be advantageous to have available an objective, unbiased method of estimating M, free from assumption as to the precise type of fundamental curve involved, but capable of taking into account more of the data than that from successive doses where p_i and p_{i+1} straddle 0.5. It would seem wise to choose such a method as *basic* (i.e., used as a basis of comparison of other methods under consideration), at least in situations where there is so little information about the form of the fundamental curve as to make any assumption about it hazardous. If calculations involved in the basic method were much simpler than those required in some rival method, then the latter should bear the burden of proof that any expectation of improvement it could offer would be worth the added effort.

The purposes of this communication are: to present such a basic method with formulas for simple direct calculation of log m as an estimate of log M, to show (in Appendix) how the variance and standard deviation of log m may be estimated from an individual assay experience in default of a broader basis for their estimation or, for purposes of comparison, to examine some of the characteristics of the present and other methods that may influence a choice among them in view of other conditions governing a given assay system, and to illustrate use of the present method in comparison with certain others on a body of data that has been presented independently for a like purpose. The proposed method is founded upon a well-known system of graduation by so-called *moving averages* followed by interpolation, all effected by use of a relatively simple formula in conjunction with a simple test of data under consideration to indicate whether the result will be an interpolation (as desired) or an extrapolation, if a given

range is used as a basis for the calculation A preliminary discussion of other methods is given below as well as an outline of test plans and sampling technics to furnish a background and foundation for development of formulas and discussion of the relative precision to be expected Random and stratified random sampling technics are outlined, that are applicable not only to quantal assays but to other assays, which may be called *gradational*

In use of methods of estimating M by fitting a curve of given type, the moderate assumption is usually implied that with a suitable limitation of range $(0 < \alpha \leqq \bar{p} \leqq \beta < 1)$ the given curve-type so nearly approximates the form of the fundamental curve as to make a satisfactory substitute under the circumstances Two curve types, the *logistic* and the *integrated normal*, appear to have been most favored Winsor (1) has shown that either may be fitted to the other so well over the ranges usually employed in bioassay that it would ordinarily be difficult to discriminate between them on the basis of goodness of fit to experimental data and usefulness as a means of estimating M

Complement-Fixation Assay Using Logistic Function von Krogh (2) used the logistic function and a transformation, $T_{(p)} = \log \dfrac{p}{1-p}$, to the coordinate system $\left(\log D, \log \dfrac{p}{1-p}\right)$ to represent the curve of hemolysis of red blood cells, finding that experimental data yielded points in the transformed system that lay approximately on a straight line,[2]

$$\log D = \log M + G \log [p/(1-p)] \tag{i}$$

Obviously, this relation is independent of the base of the logarithms, for theoretical discussions the base e is more convenient, but for calculations the base 10 may be used In any case we may convert from one form to the other by the well-known identity, $\log_e x = \log_{10} x \log_e 10 \cong 2\,30259 \log_{10} x$ Relation (i) has been used as *fundamental curve* in evaluation of bioassays (3–10, 38) that employ such hemolysis as indicator in the titration of various antigen-antibody aggregates (or either component in the presence of a maximally reactive amount of the other) by means of their complement-fixing ability under prescribed conditions—the unfixed residual complement acting as the hemolytic agent on the previously sensitized cells The total number (n) of cells used in each case is large (roughly estimated by Elizabeth and Frank Maltaner as about 125 million in their work and by Kent as about 100 million in his work with Bukantz and Rein (9)), p is estimated colorimetrically, and a restricted range for p is specified as admissible for use in evaluations

It might at first glance be supposed that the random sampling errors in p would be a dominant influence and that therefore the linear relation (i) should be fitted so as to minimize a weighted sum of squared deviations of $\log [p/(1-p)]$ from the fitted line However, by well-known relations the standard deviation of p (with all else the same in random sampling) would be $\sqrt{\bar{p}(1-\bar{p})/n}$ and twice this cannot exceed $1/\sqrt{n} \leqq 0.0001$ for $n \geqq 100$ million Accordingly, this source of error would appear to have a negligible rather than a dominant influence (with deviations of p from $\bar{p}$ numerically less than *0 0001 in nineteen*

out of twenty trials) Indeed, p is estimated in most routine tests by visual comparison with standards to the nearest 0 05 or, as in the work of Kent *et al* (9), photoelectrically to about the nearest 0 01 Errors in measuring reagents may well exert a greater influence on the results (12c) Thus the procedure they employed, minimizing the sum of squared deviations in log D, should not be rejected offhand on this basis The writer has recommended to Mr Kent, the Maltaners, and Doctor Rice, well-known methods (10a) of simplifying the calculations and of relatively simple extension to the case where a family of k parallel regression lines are fitted to k sets of data such as those obtained in the experiments of Kent *et al* (9) However, it is interesting to calculate the regression lines fitted individually to each set to see what variation in slope occurs, and likewise to compare the results of fitting of straight lines by inspection from a graph in the transformed coordinates (the technic actually used in most of the earlier work)

Now, the purpose of Kent, Bukantz, and Rein was essentially to develop more convenient and precise graphs to be used subsequently for estimation of M from the value of p obtained in a single hemolysis test with a given dose of complement D in a domain where the slope G of the regression lines (1) could be considered approximately constant This situation is realized in complement titration (where antigen, serum, and antibody are absent) and in the case of the system investigated by the Maltaners (6) in which egg albumen was used as antigen Thus relation (1) with G evaluated in previous experience could be used directly or in a graph or table to give M from the amount of complement D used and the degree of hemolysis p that was observed to result in the conventional test Kent *et al* rejected results where p lay outside the interval (0 2, 0 8) but studied relative errors both within and without this interval in formal application of their method With restriction of p thus, it appears (in their table 6) that for estimation of M the standard deviations were about 2 per cent or less Thus far we may suppose simply that M and D are expressed in the same arbitrary unit system However, it is convenient for many purposes to take the arbitrary unit so that $M = 1$ in the case of complement titration The volume of a given complement preparation that corresponds to one arbitrary unit is estimated by a preliminary or simultaneous test or both

With other antigen-antibody systems such as those designed to study reactivity of tuberculous, syphilitic, gonococcal, pneumococcal, or viral antisera with homologous antigens (4, 5, 8, 10) an additional difficulty appeared Thus it was found with the syphilitic system that with a given amount of serum and the optimal amount of antigen a relation of form (1) was approximated in all titrations with various doses D but that the slopes G of the regression lines varied greatly, and approximately systematically as a function of M The writer's first contact with these investigations was when Dr Frank Maltaner asked for help in solving the problem given a smooth graduation curve to represent the dependence of G on M, and that for a given M relation (1) holds as stated above, how can the unknown M be estimated from the degree of hemolysis p observed in a single test employing a given amount of complement D A graphic method for solving this problem was developed (7) and used to construct graphs and tables for each of the systems investigated, so that the appropriate graph or table could be entered with the given values of p and D to obtain the

required value of M directly This method may be designated briefly as the *modulation method* with the intention of suggesting that it takes into account the gradual variation in G

The original article (7) shows how ratio tables may be constructed so that data from tests with two reaction mixtures (e g., one being a *complement* or a *serum control*) and having D and p values respectively (D_1, p_1) and (D_2, p_2) may be applied to an appropriate table (for D_2, D_1) making a cross entry with p_2 and p_1 to find directly the ratio $I = M_2/M_1$ A number of tables so constructed were published (4) prior to the method itself Their form was made to accord with that customary in reporting routine results with a given specimen as the ratio I with M_2 from a test with optimal amount of antigen and M_1 without antigen Possibly this custom is only a temporary expedient pending further investigation, but it has been used because it was believed (3, 4) that to a great extent the anticomplementary activity exhibited by certain sera would have a roughly proportional effect on complement whether antigen was present or not However, the issue is not clear (38)

The egg albumen system (as has been mentioned) gave a constant G, and so did the pneumococcal system at 37° but not at 3° to 6° In that case and in all other systems investigated G was found inconstant but largely dependent on M, though differently in each, but permitting application of the modulation method Whether or not estimates of M obtained on this basis furnish a useful measure of something must rest upon demonstration with each antibody-antigen system However, all of those investigated yielded in this way values for M that were found approximately linearly related under given conditions to the relative amount of either antigen or antibody in the reaction mixture if the other agent was present in an approximately maximally-reactive amount This furnished a justification for use of the estimation method with these systems Recent investigations by Rice with the pneumococcal (8, 38) and vaccinia viral (10) systems afford examples For some time attention has been focused upon improvement of reagents as in use of the pure substance cardiolipin in tests for syphilis (41, 42)

Some estimates of reproducibility of the ratio I in routine tests have been made by constructing confidence ranges for the relative numerical discrepancy $\delta = 2(I_1 - I_2)/(I_1 + I_2)$, where $I_1 \geqq I_2$ denote replicate values of I obtained in tests with the same specimen (as in serum tests for syphilis), the replicates being either simultaneous with the same reagents or obtained on different days with different reagent preparations The statistical analysis was based on methods described elsewhere (36, 46, 47) to obtain from sample experiences values of δ_P such that the probability of encountering a value of $\delta > \delta_P$ in any given future test under the same conditions was P Estimates of $\delta_{0.5}$ and $\delta_{0.1}$ were obtained for I in different ranges in the test for syphilis and found to be nearly the same The estimate from a pooled experience with 268 pairs of nonsimultaneous observations gave $\delta_{0.5} \cong 8$ per cent and $\delta_{0.1} \cong 25$ per cent For simultaneous replicates a sample of 80 pairs gave $\delta_{0.5} \cong 5$ per cent and $\delta_{0.1} \cong 16$ per cent

Some Characteristics of Other Assay Methods A review of other literature on biologic assay has been presented by Bliss and Cattell (11) with a discussion of some of the fundamental concepts, which are also the subject of an interesting article by Irwin (12a) and discussion that followed its presentation (12b),

especially noteworthy is an appended contribution by Neyman (12c) For a given value of p, Bliss defined the *probit* as five plus the equivalent *normal deviation* (with unit standard deviation), the transformation, $T_{(p)}$ = probit p, converts the integrated normal curve to a straight line that passes through the transformed point (log M, 5), as probit 0 5 = 5 This, or the corresponding normal deviate transformation, has been applied extensively to assay systems where n is large (11, 13), and has been modified (14–16) for use where n is small (in which case p_i = 0 or 1 is not uncommonly encountered) However, in the latter case the calculations required in estimation of M are somewhat difficult, involving successive approximations with tentative regression lines fitted by a method of *maximum likelihood* The logistic function has been applied instead by Wilson and Worcester (17, 18) using maximum likelihood, and by Berkson (19, 20) using a method of weighted least squares for curve fitting In terms of the natural (base e) logarithms Berkson (19) defines logit $p = \log[(1 - p)/p] = -\log[p/(1 - p)]$, transforming to logits prior to the curve fitting (the negative of von Krogh's transformation (2)) He gives some comparisons between uses of the logistic and of the integrated normal curve in applications to the same data in a variety of situations, the comparisons uniformly in favor of the logistic Occurrence of values of p_i equal to zero or one is a source of difficulty in fitting either the integrated normal or the logistic curve to data, as the transformation to either probit or logit gives infinite values in these cases As a result, a special treatment is employed in the form of an adjustment of such data

Some attempts have been made to develop methods of estimating M that avoid the definite assumption as to the fundamental curve form and attendant difficulties of curve fitting, but emphasis has been placed mostly upon facility in calculation, most prominent have been the Kärber (21–24) and the Reed-Muench (25) methods Apparently, the most generally applicable method is the obvious device of simple interpolation between successive values of p_i that happen to straddle 0 5 This is included as a special case ($K = 1$) in the moving-average method to be presented However, this simple procedure makes no use of other assay data except in scanning, and thus in practice appears inefficient Irwin and Cheeseman (23, 24) have used data of Mrs Joyce Wilson and Professor Topley, given in the present table 1, to compare the method of Bliss (14, 15) with that of Kärber, which they call respectively "the exact" and "the approximate" method However, Kärber's method is open to several objections, it may be shown to lead, even under the most ideal conditions, to the paradoxical conclusion that the approximate median-effective dose of any toxin is *none whatever*, unless it is assumed that subjects given very small doses of toxin (or none) would bear charmed lives during the experimental interval of observation In general, it seems impossible to avoid some such vitiating feature with any suggested method that does not include an objectively applied restriction of the range of values of D_i that are allowed to influence the estimation Although any good method may be made ineffective by an inadequate experimental plan, too meager data, or extraordinarily erratic experimental results, the procedure should otherwise lead to unbiased unequivocal estimates Reed and Muench

(25) warned against effects of failure to restrict range properly, but their method has been abused regardless of the warning Furthermore, it can be demonstrated that the Reed-Muench method, even with the absolute antithesis of erratic data (a uniform trend), has an unfortunate predilection to yield biased or equivocal estimates Accordingly, neither the Kärber nor the Reed-Muench method meets the basic need for an objective, unbiased method of estimating M, free from assumption as to the precise type of fundamental curve involved, but capable of taking into account more than the data from successive doses where corresponding values of p_i straddle 0 5 Not only is this need met by the proposed moving-average method, but in simplicity of calculations it appears to be best, except that Kärber's method may be regarded as a degenerate form of the moving-average method and, accordingly, a much simpler calculation could be used instead of that traditionally employed (21, 23) Further discussion of these relations will follow a consideration of experimental plans in a quantal assay and adoption of some conventions as an aid to discussion

EXPERIMENTAL PLANS

Fundamental Curve and Sense Convention In any quantal assay system to be considered, it is assumed that the sampled population may be regarded as infinite and that $\bar{p}$, the *true* probability of critical response to the dose D in the sampled population, is either an *increasing* or a *decreasing* function of D within a convenient range of dosage including the median-effective dose M Unless otherwise stated, no other specific knowledge of the form of this function is assumed The fundamental curve is treated as if existing though unknown, formed from the hypothetical points (log D, $\bar{p}$) with log D as abscissa and $\bar{p}$ as ordinate Strictly speaking, the curve is only a fiction, convenient to use in discussion of the population sampled in any given assay test or set of tests such that the subjects may be considered as drawn from the same population

Even in use of the same colony of potential subjects for test, it may not be advisable to make such an assumption if a considerable time lapse or other possibly disturbing circumstance intervenes between tests, perhaps the fundamental curve should be considered as altered Age increase alone may alter potential susceptibility of individuals, and thus the curve may be changed To say that the fundamental curve is different implies, of course, that we are dealing with a different *sampled population* in the idealized sense intended Such possible changes in curve form through seasonal variations, generation peculiarities, and secular trends might make it practically impossible ever to have enough information about the fundamental curve to be useful in curve fitting

The critical response may be defined in any assay arbitrarily as either the occurrence or non-occurrence of a specified result to individuals (for example, death or survival), obviously without affecting the value of the median-effective dose M Accordingly, to fix the ideas, a *sense convention* will be adopted It will be assumed that the choice is so made that $\bar{p}$ is an increasing function of log D (necessarily then of D also) at least within a restricted range as mentioned above It is easily demonstrated as a check on essential formulas (and tests for interpolation) that they hold equally if q is substituted for p and vice versa and

s_i for r_i and vice versa, where $q = 1 - p$ and $s_i = n_i - r_i$ This shows the sense convention to be merely a convenience in discussion

Preliminary Conditions and Organization of Tests In any assay system a suitable choice of species, and conditions of breeding, maintenance, and preparation of *subjects* for tests are of prime importance, as well as restrictions that may be made with regard to certain attributes such as age, weight, apparent conditon, and sex, the possibilities of attainment of greater assay precision by modification of such characteristics of a test plan are discussed by Bliss and Cattell (11a) For any given set of tests wherein conditions are to be made as comparable as possible aside from such specifications, it is best to use samples in each case drawn in an unbiased manner from a pool of prospective test-subjects that have been selected as suitable in accord with the specifications Suppose that there are H ultimate categories (cases), possibly testing various reagent preparations in a given test plan, and suppose that n test-subjects are required in each case Then the pool should contain at least n H subjects, and H cells should be available, numbered 1 , , H, to correspond to the H cases so that n subjects may be placed conveniently in each cell as they are assigned by the sampling procedure Two systems of unbiased sampling are given below, based on principles and terminology discussed by Neyman (26a)

Random Sampling (Unrestricted) An aggregate of n H tags, numbered n each 1 , , H, but otherwise alike, are placed in a bowl, shuffled, and indiscriminately withdrawn one at a time without replacement as an individual is taken from the pool and assigned to the correspondingly numbered cell The term *random sampling* implies unrestricted random sampling unless otherwise indicated

Stratified Random Sampling Many systems of stratified random sampling are possible, for example, the H subjects in each stratum may be selected in ascending order of weight or other attribute from the pool, or each stratum may contain individuals alike in sex, or characterized by certain combined attributes Instead of the preceding technic, a pool of n H subjects may be initially subdivided into n arbitrary subclasses, called *strata* Only one tag for each number (1 , , H) is placed in the bowl and these are withdrawn at random, as above, without replacement as the subjects of any one stratum are assigned to cells corresponding After the random sampling of the first stratum, all tags are returned to the bowl and the sampling process is repeated successively with the other strata In the end, each cell has n subjects, one taken at random from each stratum This is called a *stratified random sample* If the strata are simply formed by the order of withdrawal of subjects from a general pool of n H or more, obviously the strata need not be preformed This may be called *simply stratified sampling*

Difficulties in probability calculations, sometimes apparently insurmountable, are introduced if an unrestricted random sample is not employed, but a stratified random sample may be preferable in some situations Thus in dealing with a pool of animals, some of which have greater ability to elude the sampler so that other less agile subjects are sooner picked for distribution in the cells, the simply stratified sampling technic would assure having

one of the first H in each cell and likewise one of each successive set of H animals so taken Such agility, if related to variation in resistance to the agent one way or another, or suspected of having some relation, might throw the weight of judgment in favor of stratification In general, the resultant precision should be as good or better if stratified rather than unrestricted random sampling is used Consideration of what forms of stratification should be tried in any given situation, and investigation of the relative merits of rival forms is a possibly important aspect of any problem of bioassay that is usually ignored entirely Even worse, a bias may be carelessly or inadvertently introduced in the sampling technic, for example, by the erroneous procedure of assigning the first n animals captured to the first cell, etc , in a fond belief that a random sampling would result

Simultaneous Comparison of Standard and Unknown The usual procedure in either quantal or gradational assay is to use a standard reagent to furnish a relative value for the unknown The standard usually should be a preparation of the same active agent, such as a standard antipneumococcus serum of the same type as another preparation that is to be tested The usually great importance of simultaneous comparisons of this sort deserves the emphasis that it has received in the literature (5a, 11, 12) It is rare that the biologic system on which the reagent acts may itself be used satisfactorily as a standard However, a striking example of this is afforded by a quantal assay employing mortality of the eggs of *Drosophila melanogaster* as a biologic indicator of x-ray dosage (43) An interesting contrast is furnished by the gradational assay system employing prolongation of the larval stage of the Drosophila by x-ray irradiation (31–33), which system affords an illustration (5a) of several types of difficulty to be encountered in either quantal or gradational bioassay

In the preceding scheme of sampling it may be supposed that usually some of the H ultimate categories (cases), corresponding to cells (cages) each containing n subjects, are to be reserved for use of the standard preparation In the case of the data to be discussed below (given in table 1), there are ten replicate assays run simultaneously with the same agent, but these are treated separately in estimation of M, as if they were all made on different preparations (or preparations not *known* to be the same) Any one of these assays (A′ to K′) could be considered as the *standard* preparation, but we need not be concerned primarily with comparison of *standard* and *unknown* for the purposes of the following discussion, which is directed toward consideration of variation in such estimates of M employing a given method, and cross-comparison of estimates obtained by different methods from the same data

The purpose is similar to that of Irwin and Cheeseman in employing the same data (23, 24) It seems obvious that such a purpose of comparison of methods applied to replicate assays would not be served by any attempt to pool information from all the assays (A′ to K′) in order to estimate some useful characteristics of the fundamental curve that might then be applied to the individual estimates However, it is hoped that the present use of a particular test plan and basis of comparison will not appear as a detraction of other methods which may permit direct comparisons of relative potency (possibly without reference to median-effective dose) or abbreviation of tests as in the complement-fixation systems discussed above and in other assay systems (45)

DEVELOPMENT OF FORMULAS FOR INTERPOLATION FROM MOVING AVERAGES

Now, consider certain formal relations that lead to a development of formulas that yield by simple calculations an estimate m of the median-effective dose M Suppose we have data in the previously indicated form of associated values of D_i and r_i for a given number n_i of subjects in each case, usually with $n_i = n$ resembling any column A′ to K′ in table 1 Assume that these have been obtained in accord with a suitable test plan with a set of h doses (D_i) wherein $D_j = R \cdot D_{j-1}$ and R is a constant greater than one $(i = 1, \quad, h, j = 2, \quad, h)$

TABLE 1

*Deaths (r_i) in ten differently labeled samples of mice injected with given doses (D_i) of the same toxin preparation** $L_i = \log D_i$

i	L_i	D_i	A′	B′	C′	D′	E′	F′	G′	H′	J′	K′
		mg										
1	$\bar{2}$ 7959	0 0625	1	1	0	2	0	0	0	1	0	1
2	$\bar{1}$ 0969	0 125	2	2	0	0	0	0	0	3	0	0
3	$\bar{1}$ 3979	0 25	3	1	5	5	3	2	4	2	3	5
4	$\bar{1}$ 6990	0 5	5	5	4	5	4	1	3	5	3	4
5	0 0000	1 0	5	4	4	5	5	5	5	5*	2	5
6	0 3010	2 0	5	5	5	5	5	5	5	5	5	5
7	0 6021	4 0	5	5	5	5	5	5	5	5	5	5

* Note Data of Wilson and Topley used by Irwin and Cheeseman (23) In another article Irwin and Cheeseman (24) give the same table except for an apparent typographic error giving 2 instead of 5 for the third entry from the bottom and right Five animals were used in each case ($n = 5$), taken at random from 350 male mice from normal stock (weights between 28 and 32 grams) Ten replicate sets (labeled as indicated at the column heads, except that primes have been added here to avoid confusion with other symbols) were injected in each case (i) Any one of the columns (A′ to K′) corresponds to an assay test The composite test-plan has $h = 7$, $H = 10$ $h = 70$, $n = 5$, n $H = 350$, $R = 2$, and $d = \log 2 \cong 0$ 30103 The critical response was death within a definite period of observation (four days after injection)

Thus the doses form a geometric progression, and their logarithms form an arithmetic progression with a constant difference between successive values that we will represent by d For convenient abbreviation, let

[1] $\qquad L_i = \log D_i$, and, accordingly,

[2] $\qquad d = L_j - L_{j-1}$ for $j = 2, \quad, h$, whence $d = \log R$ and

$$L_{i+u} = L_i + d \cdot u$$

Subscript Convention For the sake of brevity, in the preceding and other expressions where the implied meaning is obvious, let any symbol used in a subscript without an accompanying fuller definition be allowed any meaning consistent with prior definitions of other symbols in the relations involved Thus obviously, u, above, is an integer greater than $-i$ and not greater than $h - i$ Accordingly also, it is unnecessary above to define j by the expression $j = 2, \quad, h$, indeed, i could have been used instead of j throughout, as the equations above in which j is employed as a subscript are obviously intended to indicate relations between things already defined, not to define something denoted by L_0 nor D_0

Common (base 10) logarithms are intended in these relations unless otherwise implied However, it may sometimes appear convenient to use logarithms to the base R instead, as then $d = \log_R R \cong 1$, and if one of the values of L_i is an integer in this system then all are integers in a natural succession Thus in figure 1 the data of table 1 are represented graphically by circled points with $p_i = r_i/n_i$ as ordinate and $\log_2 D_i$ as abscissa for $i = 1, \quad , h$ in each of ten diagrams corresponding to the columns A′ to K′ of the table

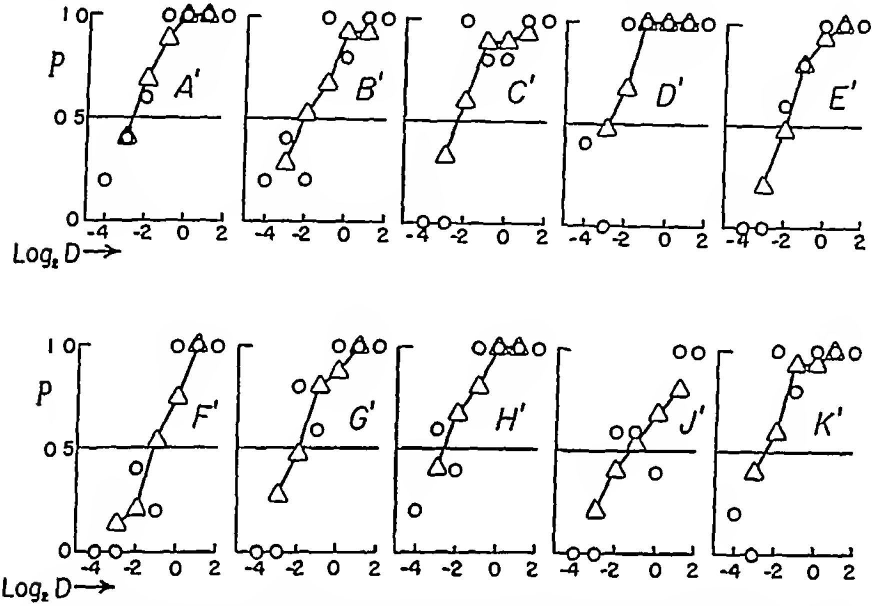

FIG 1 Illustration of Moving Average Estimation of Median Effective Dose The circles represent $p_i = r_i/n$ from the data of table 1, the triangles are corresponding moving average of three successive values of p_i, their join lines form a polygonal curve that crosses the 0 5-horizontal at the *median point* estimate, whose abscissal value is $\log_2 m$ The mean $\log_2 m \cong -2\ 123$ and the standard deviation estimate $s' \cong 0\ 588$, corresponding to a per centage deviation in m of -33 or $+50$

Illustration of Graduating Influence Obviously, an attempt to estimate the median-effective dose M by simple interpolation between the circled points of figure 1 would lead to erratic and occasionally equivocal results, as may be seen by joining successive circles by straight lines in each graph If instead we take the triangle-enclosed points in these graphs, which have respective arithmetic means of three successive values of p_i as ordinate and the mean of the three corresponding values of L_i as abscissa, the join-lines as given in the graphs indicate an *ironing out* of some of the erratic variations Where such a join-line crosses the $p = 0\ 5$ horizontal, also illustrated, the intersection abscissa is $\log m$ which is the estimate of $\log M$ in this case (for moving averages of *spans* of three successive values of p_i) However, calculation of $\log m$ algebraically is preferable and even simpler

General Notions of Moving-Average Interpolation. General formulas for interpolation from moving-average points for spans of K successive values of p_i are readily developed from definitions of successive points designated by (L', p') and (L'', p''); actual graphic constructions are not required. For brevity let $b \equiv a + K$, and let

[3]
$$p' = \frac{p_a + \cdots + p_{b-1}}{K}, \text{ and}$$
$$p'' = \frac{p_{a+1} + \cdots + p_b}{K},$$

and correspondingly let

[4]
$$L' = \frac{L_a + \cdots + L_{b-1}}{K} = \log D', \text{ and}$$
$$L'' = \frac{L_{a+1} + \cdots + L_b}{K} = \log D''$$

If $p' \neq p''$ and $p' \leqq 0.5 \leqq p''$, then it is possible to estimate $\log M$ (the value of $\log D$ for $\bar{p} = 0.5$, as previously defined) by a simple linear interpolation, as follows.

Let $\log m$ denote this estimate, then

[5]
$$\log m = L' + d\,f, \text{ where } f = \frac{0.5 - p'}{p'' - p'}$$

Calculation of $\log m$ may be simplified by use of general formulas, or simplified formulas in given situations.

General Formulas for log m. Obviously, relations [2] and [4] give $L' = L_a + d\,(K - 1)/2$, whence relations [3] and [5] give the general formula,

$$\log M \cong \log m = L_a + \frac{d(K-1)}{2} + d\,f, \text{ where}$$

[6]
$$f = \left(\frac{K}{2} - p_a - \cdots - p_{b-1}\right) \Big/ (p_b - p_a) \quad \text{for } p_i = r_i/n_i,$$

which reduces to
$$f = \left(\frac{nK}{2} - r_a - \cdots - r_{b-1}\right) \Big/ (r_b - r_a) \qquad \text{for } n_i = n$$

Obviously, from the definition in relation [5], the result is an interpolation if and only if the fraction f lies in the unit interval $(0 \leqq f \leqq 1)$. This is equivalent in the latter form (for $n_i = n$, the usual condition of the test plan) to the requirement that $r_b \neq r_a$ and that the *r-trial* function defined as $r_a + \cdots + r_b - (nK/2)$ lie in the interval (r_a, r_b), and it is convenient to run down the conventional column of r-values, summing *comprehensive spans* of $K + 1$ successive

values and noting when this sum minus one-half nK is equal to the first or last value of r in the summation or between them. Then the index of the first value of r *is a in the formula above*.

Where the values of n_i are not all alike, a corresponding test (valid in general) may be made that $p_a \neq p_b$ and the *p trial* function, $p_a + \cdots + p_b - K/2$, lie in the interval (p_a, p_b) is a necessary and sufficient condition for the result of application of formula [6] to be an interpolation. It should be noted that the *comprehensive* (total) *index span* of data used for the estimation is $K + 1$, one more than the moving average span K.

As mentioned previously, it is readily verified that transposition of p_i and q_i and of r_i and s_i in formula [6] gives the same value of $\log m$. The trial functions and corresponding intervals used in the test for interpolation may be altered but correspondingly so that the test yields the same conclusion. Accordingly, here as will be found elsewhere, the *sense convention* about the fundamental curve is only an aid to discussion. Furthermore, d need not be positive, and values of D_i may be replaced in the protocol by values proportional to their reciprocals (for example, by a corresponding sequence of dilutions of reagent) provided that proper interpretation of the formal result is made. However, the conventional meanings will be retained for the sake of clarity.

Simplification of Formulas in Given Situations. It seems advisable to fix K on the basis of some experience with a given system, as well as n, d, and h in the test plan, with care to reduce to a tolerable risk the chance of obtaining results that are indeterminate, although there always is some risk of this with any method of estimating median-effective dose from data of the kind considered. Once any of the parameters d, n, and K have been fixed, relation [6] may be simplified further by substitution of the given constants in the formula, and usually also by a collection of some terms.

Thus with the data of table 1, where $n = 5$ and $d = \log 2 \cong 0.30103$, if we decide to use $K = 3$ as illustrated graphically in figure 1, then we may first simplify relation [6] to

$$[6.1] \qquad \log m = L_a + d\left(\frac{3-1}{2}\right) + d\left(\frac{7.5 - r_a - r_{a+1} - r_{a+2}}{r_{a+3} - r_a}\right),$$

$$[6.2] \qquad \log m \cong L_a + 0.30103\left(1 + \frac{7.5 - r_a - r_{a+1} - r_{a+2}}{r_{a+3} - r_a}\right)$$

Correspondingly, the trial function is $r_a + r_{a+1} + r_{a+2} + r_{a+3} - 7.5$ under the circumstances, and its value must lie in the interval (r_a, r_{a+3}) with $r_a \neq r_{a+3}$ for the result to be an interpolation, and this is also a sufficient condition.

Example Calculations and Use of Trial Function. Consider the data of Column A′ of table 1. To try the suitability of $a = 1$ for interpolation by formula [6.2], we evaluate the trial function as $\underline{1} + 2 + 3 + \underline{5} - 7.5$ which equals 3.5, and as this lies in the interval from 1 to 5 (the first and last of the successive values of r in the summation just given) we know that an interpolation will be the result. It is readily verified that any value of a greater than unity would not satisfy the trial-function test in Column A′. Next, taking $a = 1$, we substitute the required values of r in formula [6.2] which gives, as $L_a = L_1 \cong \bar{2}.7959$ from the table,

$$\log m \cong \bar{2}.7959 + 0.30103\left(1 + \frac{7.5 - 1 - 2 - 3}{5 - 1}\right)$$

$$\cong \bar{2}.7959 + 0.30103(11/8) \cong \bar{2}.7959 + 0.4139 \cong \bar{1}.210$$

Now, consider Column E′ Successively test $a = 1, 2,$, etc With $a = 1$ the trial function value is $\underline{0} + 0 + 3 + \underline{4} - 7.5 = -0.5$, obviously not in the interval (0,4) Trial of $a = 2$ gives instead $\underline{0} + 3 + 4 + \underline{5} - 7\,5 = 4.5$, which *is* in the interval (0, 5) Trial of $a = 3$ gives $\underline{3} + 4 + 5 + \underline{5} - 7\,5 = 9.5$, which is outside the interval (3, 5) Obviously, greater values of a will not meet the test condition, and use of $a = 2$ is the only procedure to yield interpolation by application of formula [6.2] to E′ Accordingly, as $L_2 = \bar{1}\,0969$, we obtain the estimate

$$\log m \cong \bar{1}\,0969 + 0\,30103\left(1 + \frac{7\,5 - 0 - 3 - 4}{5 - 0}\right)$$

$$\cong \bar{1}\,0969 + 0\,30103(11/10) \cong \bar{1}\,0969 + 0\,3311 = \bar{1}\,428$$

Estimation of Variance and Standard Deviation In general (27, 28) for any variate X, if we have a random sample of N values (X_j), where $j = 1,$, N, we denote the sample mean by $\bar{X}$ and let v_X be the estimate of variance of X in the sampled population, where $\bar{X} = (1/N)\ \Sigma X_j$ and $v_X = \Sigma\,(X_j - \bar{X})^2/(N - 1)$ The *true* variance of X in the sampled population is denoted by σ_X^2 and the standard deviation is its square root, $\sigma_X \geqq 0$ We let $s'_X = \sqrt{v_X}$ represent the estimate of standard deviation (the notation being in accord with Rider's text (28))

From Columns A′ to K′ of table 1 we obtain with $K = 3$, as indicated previously, the following ten estimates of log M successively log m = $\bar{1}.210$, $\bar{1}$ 360, $\bar{1}.285$, $\bar{1}.147$, $\bar{1}$ 428, $\bar{1}$ 669, $\bar{1}$ 428, $\bar{1}.210$, $\bar{1}$ 624 and $\bar{1}.247$ The mean value is approximately $\bar{1}$ 361, and the standard deviation of log m estimated by $s'_{\log m} \cong 0.177$

Another method of estimating $\sigma_{\log m}$ where extensive replicate assays are not available is derived and discussed in the Appendix The estimate may be made from data of a single assay, but it is strictly applicable only where subjects are obtained by unrestricted random sampling Nevertheless, it furnishes an approximate indication of any gain or loss in efficiency by use of other sampling technics, for example, if stratified random sampling technic is used, it should lead to greater, if not equal efficiency (26a)

FEATURES OF MOVING-AVERAGE INTERPOLATION IN RELATION TO OTHER METHODS

Comparison of Results by Other Statistical Methods The data of Wilson and Topley in table 1 furnish a ready basis for comparing estimations of log M by certain formulas such as relation [6] with the results Irwin and Cheeseman obtained (23, 24) from the same data by the method of Bliss (14, 15) and by that of Kärber (21, 22) By Bliss's method they estimated log (LD_{50}), log M in the present notation, and obtained a mean value of $\bar{1}$ 361 and standard deviation estimate $s' = 0\,199$ The results by the present method with a moving-average span $K = 3$ compare favorably mean $= \bar{1}\,361$ and $s' = 0\,177$ The results by these and two other methods are indicated in table 2, but, for convenience, 1000 times the difference between the estimate of log M and $\bar{1}$ 361 is listed in each instance Results by Kärber's method and by that of Reed and Muench are

thus given With the latter an abridgement to use of a total index span of four was made, in order to correspond to use of $K = 3$ for the moving-average method For each assay (A′ to K′) the relative order of absolute deviation from $\bar{1}.361$ is given in parentheses for the results obtained by the four rival methods, or mean values in cases of a tie The mean of these order-numbers for each method is given also parenthetically at the base of the table

TABLE 2

Differences times 1000 between estimates of log M and the arbitrary reference point, $\bar{1}.361$, for the ten replicate assays of table 1 by different methods The four estimates for each assay are given order numbers in parentheses according to absolute deviation from the reference point Results by the first two methods are those of Irwin and Cheeseman (23, 24)

ASSAY LABEL	METHOD			
	Bliss	Kärber	Reed Muench (4-span)	Moving Average ($K = 3$)
A′	−190, (4)	−174, (3)	−164, (2)	−151, (1)
B′	−20, (3)	+7, (2)	+37, (4)	−1, (1)
C′	+30, (2)	+7, (1)	−83, (4)	−76, (3)
D′	−262, (4)	−234, (3)	−214, (1 5)	−214, (1 5)
E′	+77, (4)	+ 67, (2 5)	+37, (1)	+67, (2 5)
F′	+295, (1)	+308, (2 5)	+376, (4)	+308, (2 5)
G′	+79, (4)	+67, (2 5)	−1, (1)	+67, (2 5)
H′	−199, (4)	−174, (2)	−189, (3)	−151, (1)
J′	+311, (4)	+308, (3)	+282, (2)	+263, (1)
K′	−120, (4)	−114, (2)	−114, (2)	−114, (2)
Mean	0, (3 4)	+7, (2 35)	−3, (2 45)	0, (1 8)
s'	199	190	198	177

Use of $\bar{1}.361$ as arbitrary reference point in computation of the deviations might be suspected of placing the other methods in an unfavorable light, but this was the mean obtained by the moving average and the Bliss methods The order numbers would be the same if the grand mean estimate, $\bar{1}.362$, were used instead or if the mean, $\bar{1}.358$, from the results with the Reed-Muench method were used If the mean from Kärber's method, $\bar{1}.368$, were taken as reference point instead, this would affect the order numbers only in an interchange of one and two in the row for B′, and the mean orders correspondingly by 0 1 as an increase for the moving average method and decrease for that of Kärber

Absolute deviations of estimates by the Bliss and moving average methods for the same assay (A′ to K′) are nowhere equal and favor the Bliss method only twice in the ten trials On the *null hypothesis* that the probability is one half that the Bliss method would deviate less on any given trial than would the moving average interpolation (equality excluded), that this should occur no more than twice in ten independent trials would have a probability $P = 56/1024 < 0\ 055$ This is almost at the ordinary critical level of significance ($P = 0\ 05$), moreover, the *burden of proof* in this situation lies on the opposite side Thus the evidence of Wilson and Topley's data would not warrant use of Bliss's method instead of the *basic* (moving-average) method in the situation in question

Comparison in Principle with Kärber's Method Irwin and Cheeseman (23, 24), referring to the Bliss method as "the exact" and to Kärber's as "the approxi-

mate" method, used the latter in quest of a less difficult means of estimating log M, yet they conformed to precedent in use of an unnecessarily complicated computation procedure suggested by Kärber (21) As shown below, the same result may be obtained more simply However, it appears essential for consistent use of the method to make a disagreeable assumption about the biological system beyond the actual range of observations, namely, that for any dose D_i outside the experimental range on one side $\bar{p}_i \equiv 0$ and on the other side $\bar{p}_i \equiv 1$ By the *sense convention* this would mean $\bar{p}_i \equiv 0$ for $D_i < D_1$, and $\bar{p}_i \equiv 1$ for $D_i > D_h$

Tentatively suspend judgment of acceptability of the assumptions and disregard the issue of interpolation or extrapolation Then, in the present notation the prerequisite assumption of Kärber's method (for $n_i = n$ as usual) may be stated as follows there exist numbers, α and β, such that $r_i = 0$ for $i \leqq \alpha$ and $r_i = n$ for $i \geqq \beta$, furthermore i may be considered as extended indefinitely in either direction with the corresponding dosage values defined by the relation, $D_i \equiv R^i D_0$ Then, for $a \leqq \alpha$ and $b \geqq \beta$, we have $r_a = 0$ and $r_b = n$ Now, for this case in relation [6] we have

$$\text{[7]} \quad \log m = L_a + d(K - \tfrac{1}{2}) - \frac{d}{n}(r_{a+1} + \quad + r_{b-1}), \qquad \text{if } r_a = 0 \text{ and } r_b = n,$$

whence, as $b \equiv a + K$, relation [2] gives

$$\text{[8]} \quad \log m = L_b - \frac{d}{2} - \frac{d}{n}(r_{a+1} + \quad + r_{b-1}), \quad \text{if } r_a = 0 \text{ and } r_b = n$$

It is readily verified that this formula yields, with an indicated simpler computation, identically the same result as the method of Kärber (21–24) However, as the risks of extrapolation are disregarded, Kärber's method appears as a degenerate form of the moving-average method, even if the special assumptions are not challenged

It is objectionable under some circumstances to extend K, the moving-average index span, more than enough to provide a stabilizing influence on the estimates of log M This is discussed below and indicated by results in table 3 With regard to the prerequisite assumption of Kärber's method, it is obvious by use of relation [2] and simple rearrangement of terms that with any $a \leqq \alpha$ and $b \geqq \beta$ in relation [8] the same value of log m is obtained Thus, provided this assumption is made, further extension of the index span ($K = b - a$) makes no difference in the result However, the extension may be so great in any case that only extrapolation is possible, actually so six times in ten with the data chosen for illustration by Irwin and Cheeseman (23, 24) as indicated in the last column of table 3

A questionable common practice in actual use of Kärber's method if $r_1 \neq 0$ is to take $a = 0$ assuming $r_0 = 0$, or likewise if $r_h \neq n$ then to take $b = h + 1$ assuming $r_{h+1} = n$ Objection may be raised against an assumption that values of r_i for lower doses of toxin than used in the actual experience would all be zero in a hypothetical extension of the experience in order to provide one of the conditions necessary to application of Kärber's method In the situation presented in table 1, this would amount to an assumption that a

temporary (four-day) immortality would have been conferred upon any and all animals injected in the prescribed way with a dose less than the least that was actually used On the other hand, if $\bar{p}_i$ approaches a constant > 0 as i decreases indefinitely, use of Kärber's method (formally equivalent to use of relation [8]) with extension of the experience indefinitely to include actual use of lower and lower doses D_{1-j} for $j = 1$, , etc , and taking a successively lower should lead to lower and lower estimates of log M with m approaching zero as a limit Otherwise stated, the median-effective dose of any toxin would always be estimated as no toxin at all It seems thus that an obviously absurd result is avoided in practice only by failure to extend the actual experience to low enough doses to make the great potential influence of the sum, $r_s + r_{s-1} + \quad + r_{s-j} + \quad$, apparent Such a

TABLE 3

Differences times 1000 between estimates of log M and the arbitrary reference point, Ī 361, for the ten replicate assays of table 1 by the method of moving averages with various index spans K, showing bias introduced with extrapolations and a counter distorting effect of substituting $r_1 = 0$ instead of the known values in the case of K = 6, an exaggeration of influences in Kärber's method as used by Irwin and Cheeseman (23, 24) which is equivalent here to use of K = 7 with the assumption that $r_0 = 0$ is applicable beyond the range of the experience

K⟶ / Assay Label	1	2	3	4	5	6	6 ($r_1 = 0$?)	7 ($r_1 = 0$?)
A′	−114	−114	−151	−189′	−226′	−264″	−114′	−174′
B′	+150	+87	−1	−13	−1′	−38′	+67′	+7′
C′	−114	−114	−76	−38	+7′	+7′	+7′	+7′
D′	−114	−114	−214	−314′	−415″	−515‴	−114′	−234′
E′	−13	+37	+67	+67	+67	+67′	+67′	+67
F′	+451	+388	+308	+308	+308	+308	+308	+308
G′	−76	−13	+67	+67	+67	+67′	+67′	+67
H′	−126*	−114	−151	−189′	−226′	−264″	−114′	−174′
J′	+338*	+288*	+263	+308	+308	+308	+308	+308
K′	−114	−114	−114	−114	−151′	−189″	−53′	−114′
Mean	+19	+22	0	−11	−26	−51	+43	+7
s'	213	183	177	206	233	261	159	190

Note An asterisk indicates that a unique result was not obtained, the mid-range of the calculated values is given The single (′), double (″) and triple (‴) primes after numbers indicate that the estimate was not found within the interpolation interval, but the shortest possible extrapolation was used, requiring extension respectively to the first, second or third interval beyond

dilemma appears to confront any attempt to base a method of estimating median-effective dose upon such assumptions about the *true* probability of critical response $\bar{p}_i$ beyond the range of experiment It appears necessary to provide some equivalent of a limitation of the range of values of D_i that are allowed to influence the estimation, objectively applied rather than as a fortuitous result of an obvious practical need for some limitations in any experiment

Extent of Moving-Average Span (*K*) The results given in table 3 serve to illustrate the influence of choice of the index span of moving averages, taking $K = 1, \quad, 6$ For convenience, as previously, 1000 (log m − Ī 361) is listed

instead of log m Use of $K = 1$ amounts to the same thing as simple interpolation between successive values of p_i that straddle 0 5, and whenever there is more than one such pair equivocal results are obtained These occurred in two of the ten replicate assays (H′ and J′), they are indicated in the table and are apparent on inspection of the circled points of figure 1 Where a unique result is not obtained, the midpoint between extremes of the equivocal values is given in the table as indicated by an asterisk With $K = 2$, the least span for actual use of moving-average graduation, only one assay (J′) gives equivocal values With $K = 3$ the results are all unique interpolations If we take K greater than this then estimation of log m is sometimes beyond the reach of interpolation from the available data (table 1), but the least possible extrapolation is used This is indicated in table 3 roughly by single, double or triple primes to signify respectively that extrapolation was required involving a reach to the first, second, or third equal interval (of length d) beyond that corresponding to interpolation With $K = 4$ such effects are of a relatively minor nature, and are encountered in only three of the ten assays (A′, D′, and H′) As K is increased beyond four, extrapolation is required more frequently and appears to be more influential in introducing bias into the mean estimates of log M, a trend toward lower mean values of log m is noticeable Precision, as indicated by the standard deviation estimates s', also seems best with $K = 3$

Extended K and Assumptions of Kärber's Method If the assumption were correct, as made by Irwin and Cheeseman in applying Kärber's method to the data of table 1, that smaller doses such as $D_0 = 0$ 03125 mg of the toxin preparation would have yielded no deaths in response and thus $r_0 = 0$ in every assay (A′ to K′), then we might use $K = 7$ as we have already used $K = 1$, , 6 The results thus formally obtained by use of the reduced form of relation [6] given in relation [8] for the special case ($r_a = 0$, and $r_b = n$) are necessarily the same as those obtained by Kärber's method, but are given again (table 3) to emphasize a failure to find in this last member ($K = 7$) evidence of family traits apparent in the six preceding offspring A sudden reversal of the trend in mean log m is a striking result having successively, with $K = 1$, , 6, obtained mean values of 1000 (log m − $\bar{1}.361$) = +19, +22, 0, −11, −26, −51, we obtain the value, +7, for $K = 7$ with the questionable assumption that we should have found $r_0 = 0$ throughout if D_0 had been used There is a corresponding reversal of trends in frequency and extent of extrapolation and in the estimate s' of standard deviation, all ostensibly favorable but damningly contrary to evident family traits In the next to last column of table 3 are presented results of an exaggeration of the suggested influence, obtained with $K = 6$ by substituting zero for the actual values of r_1 which is equivalent to assumption that $r_1 = 0$ throughout in ignorance of the actual data (obviously false five times in ten)

The evidence suggests that apparently good results obtained by Kärber's method may be due to compensating errors of the sort introduced in the present case by a subjective judgment that r_0 should be zero and by use of an excessive index span ($K = 7$)

Characteristics of Indefinite Cumulant and Reed-Muench Methods Objections, closely resembling those made to Kärber's method, appear to discourage any attempt to base methods upon two indefinite cumulants, the sum of all values of r_i for $i \leqq c$ and the sum of all values of s_i for $i \geqq c$

Briefly these sums may be denoted by $\sum^{c} r$ and $\sum_{c} s$ where

[9] $$\sum^{c} r = \sum_{i=1}^{c} r_i \quad \text{and} \quad \sum_{c} s = \sum_{i=c}^{h} s_i$$

provided it is assumed that $r_i = 0$ for $i < 1$ and that $s_i = 0$ for $i > h$ The methods employ

[10] $$\phi_c' = \sum^{c} r / (\sum^{c} r + \sum_{c} s) \text{ and } \phi_c'' = \sum^{c'} r / (\sum^{c'} r + \sum_{c'} s) \text{ where } c' = c + 1$$

to obtain an estimate m' of the median-effective dose M by linear interpolation between points, $(\log D_c, \phi_c)$ and $(\log D_{c'}, \phi_c'')$, to find the "endpoint", $(\log m', 0\ 5)$ It is noteworthy that here $\phi_c'' = \phi_{c'}'$ Obviously, the results of this procedure could be greatly biased by not having $n_i = n$, a constant, although substitution of p_i for r_i and q_i for s_i throughout would remove this objection

Reed and Muench (25) adopted the condition $n_i = n$, and specified that the data be abridged so that the calculation of the "endpoint" is based on data from an equal number of dosage values (D_i) on each side of it This specification is essentially equivalent to limiting the range of the index i in the summations to the inclusive interval $(c + 1 - k, c + k)$ for an arbitrary integer k, provided that c is such that $\phi_c' \leqq 0.5 \leqq \phi_c''$ Strictly interpreted, the equality signs in the last expression admit an exception to the provision of Reed and Muench, " an equal number of dilutions is taken on each side of the endpoint " However, their statement (25) is immediately followed by an example that appears to condone even further departure from a strict interpretation of the provision, although the data there given would have yielded the same result either way

The Reed-Muench method appears to be placed in the most favorable light on the suggested basis, which may be given explicitly as follows

[11] $$\phi_c' = \frac{r_{c+1-k} + \quad + r_c}{r_{c+1-k} + \quad + r_c + s_c + \quad + s_{c+k}}, \quad \text{and}$$

$$\phi_c'' = \frac{r_{c+1-k} + \quad + r_{c+1}}{r_{c+1-k} + \quad + r_{c+1} + s_{c+1} + \quad + s_{c+k}},$$

and the median-effective dose M is estimated by the "endpoint" value m', given by

[12] $$\log m' = \log D_c + d\ \frac{0\ 5 - \phi_c'}{\phi_c'' - \phi_c'}, \quad \text{provided} \quad \phi_c' \leqq 0\ 5 \leqq \phi_c''$$

Thus, if the range of the index i is great enough in the protocol, for a given value of k there may be determined a succession of intervals, (ϕ_c', ϕ_c''), one of which may be found to contain 0 5 and thus serve in evaluation of m' in relation [12] Unfortunately, the successive intervals, (ϕ_c', ϕ_c'') and $(\phi_{c'}', \phi_{c'}'')$ where $c' = c + 1$, overlap except in the case where $r_{c+1-k} = s_{c+1+k} = 0$ and in two *trivial* other cases where ϕ_c'' and $\phi_{c'}'$ are both zero or both one This is readily verified from the definitions in [11] Thus there is an almost universal tendency for the Reed-Muench method to give biased or equivocal results, the latter occurring whenever more than one of the successive intervals contains 0 5

A simple example is afforded by consideration of a uniform trend, the absolute antithesis of erratic data Thus suppose that $r_i = i$ for $i = 1, \quad, n - 1$, and $s_i = n - r_i = n - i$ In this hypothetical assay suppose that the doses are successively doubled $(d = \log 2)$

Let M' be the geometric mean of the doses (D_i) for $i = 1, \quad , (n - 1)$, then apparently M' is the best estimate of M that can be made in this situation on any basis This is the value that would be given by the moving-average method with any value of the index span K and interpolation (or even extrapolation from any admissible part of the data) The same result would be obtained from all the data by the methods of Bliss, Berkson, and Kärber (provided that in the last method we use $r_0 = 0$ and $r_n = n$, in extension of the data) However, the Reed-Muench method gives equivocal estimates (m') The simplest way to present the results is to give the ratio $g' = m'/M'$ Thus for a total index span $2\lambda = 4$ we have if $n = 10$, then equivocally $g' \cong 0.823$ or 1.215, and if $n = 15$ then $g' \cong 0.617, 1.000$, or 1.621 Similarly, for a total index span $2\lambda = 6$ we have if $n = 10$, then $g' \cong 0.878$ or 1.138, and if $n = 15$, then $g' \cong 0.683, 1.000$, or 1.463 The purpose here, of course, is to illustrate the character of the equivocal results, not to indicate the magnitude of relative differences to be expected in actual use

In application of the Reed-Muench method to any actual data, following the procedure indicated by the authors, more than one value for m' would not ordinarily be obtained, because the first one found would be accepted A limitation placed experimentally upon the range of values of D_i might prevent recognition of the danger, just as might ignoring part of a more extensive set of results (deleting data for successive dosage values at either the top or bottom of the conventional protocol) Obviously, this would be done in either case at the expense of introduction of some bias according to what range of doses is allowed to influence estimation of the median-effective dose M, the estimate m' tending to be too near the geometric mean of the range of doses so employed Replicate assays made with the same range of doses would tend to develop in the observer a false confidence in estimates as a result of apparent precision, if he were not aware of this artificial centrifugal tendency in m' Hedén (49), in a recent article suggesting application of the Reed-Muench method to serologic titrimetry, has given a table in which he cautions against some of the most prominent points of danger

Road to Further Modifications of Cumulant Method It is evident that the Reed-Muench modification of the cumulant method evokes new defects in place of those it removes The new sources of difficulty are a failure in definition to make ϕ_c'' identical with ϕ_{c+1}', and to have ϕ_c' such that the range of the index values involved extends equally far to each side of c, if D_c is taken as the associated dosage value used in the interpolation D_c would then be the geometric mean of dosage values corresponding to values of r_i and s_i involved in the definition of ϕ_c' This would avoid the vicious overlapping and any obvious tendency toward biased results However, it seems preferable to define the cumulants and ϕ_c' in terms of p_i and q_i rather than r_i and s_i, for the sake of greater generality, the other forms are readily obtained where $n_i = n$ throughout by substitution of the identical values, $p_i = r_i/n$, and $q_i = s_i/n$,

Thus, we might redefine the bases of interpolation, ϕ_c' and ϕ_c'', by

$$[13] \qquad \phi_c' = \frac{p_{c+1-k} + \quad + p_c}{p_{c+1-k} + \quad p_c + q_c + \quad + q_{c-1+k}}, \quad \text{and} \quad \phi_c'' = \phi_{c+1}'$$

The "endpoint" value m' could be defined by relation [12] in these new terms

However, this is not the only possible recourse Indeed, it is not essential to a use of cumulants that ϕ_c' be a cumulant ratio as in [10], [11], or [13], but we should agree to the condition that henceforth $\phi_c'' = \phi_{c+1}'$ *identically* In the last case [13] the underlying idea is, in accord with that of other cumulant methods, to find or estimate by some form of interpolation a dose that should be expected to yield a zero cumulant difference, i e, the

p-cumulant minus the q-cumulant should be zero This suggests redefinition of ϕ_c' as some convenient linear function of the cumulant difference, $p_{c+1-k} + \cdots + p_c - (q_c + \cdots + q_{c-1+k})$ Furthermore, as $q_i = 1 - p_i$ identically, we note that

$$[14] \quad p_{c+1-k} + \cdots + p_c - (q_c + \cdots + q_{c-1+k}) = p_{c+1-k} + \cdots + p_{c-1} + 2p_c + p_{c+1} + \cdots + p_{c-1+k} - k$$

Accordingly, it would appear convenient to *redefine*

$$[15] \quad \phi_c' = \frac{p_{c+1-k} + \cdots + p_{c-1} + 2p_c + p_{c+1} + \cdots + p_{c-1+k}}{2k}$$

As agreed, ϕ_c is defined as identical with ϕ_{c+1}' Interpolation formally by relation [12] gives the same "endpoint" as that for a cumulant difference of zero as suggested above

Formula [15] may be recognized as that for a weighted moving average, many different forms of which have been employed as graduation formulas (29a) It differs from the simple moving average designated by p' in relation [3] only in giving double weight to p_c in the middle term of the numerator, and in a limitation to use of odd index spans, $K = 2k - 1$ The denominator, of course, is the sum of the weight coefficients If we define

$$[16] \quad \theta = \frac{(k - p_{c+1-k} + \cdots + p_{c-1} + 2p_c + p_{c+1} + \cdots + p_{c-1+k})}{p_{c+k} + p_{c+1} - p_c - p_{c+1-k}},$$

then the interpolation (provided $0 \leq \theta \leq 1$) is given by

$$[17] \quad \log m'' = \log D_c + d\,\theta,$$

where m'' is the estimate of the median-effective dose M It should be noted that the test for interpolation and the calculation are more difficult than in use of the simple moving-average formula [6] Furthermore, the restriction to use of an odd index span ($K = 2k - 1$) is awkward, and no compensating advantage is apparent

An Equivalence of Simple Moving-Average and Modified Cumulant Methods If we take a further modified cumulant difference, $p_{c+1-k} + \cdots + p_c - (q_{c+1} + \cdots + q_{c+k})$, and associate this with the geometric mean of dosage values involved in obtaining these data (which is equivalent to what was done in the preceding modifications), then this dosage value is readily found to be $\sqrt{D_c\, D_{c+1}}$ Then, proceeding in steps analogous to those leading from relation [14] to [16] and [17], we are led to relations identical with formula [6] *for the simple moving-average method except that* $K = 2k$ *and thus is always even* Now, if we take another modification of cumulant difference, $p_{c+1-k} + \cdots + p_{c-1} + \frac{p_c}{2} - \frac{q_c}{2} - (q_{c+1} + \cdots + q_{c-1+k})$, and associate this with the geometric mean dose involved (D_c), again we are led by analogous steps to the same formula [6], but this time find that the index span is always *odd*, $K = 2k - 1$ Thus it appears that a process of successive modification of cumulant methods, in attempts to remove evident defects and avoid unjustified awkwardness, leads directly to the simple moving-average method that has been proposed above on the basis of immediate intuitive appeal

The approach from the point of view of cumulant *differences* lies along an easily followed trail as far as the next to last modification There the appearance of slight difficulty in the associated dosage value ($\sqrt{D_c\, D_{c+1}}$) might have discouraged further progress as might

the apparent introduction of complications with the companion modified cumulant difference, but, only a little further along, all such difficulties are left behind as we arrive at the same situation previously attained by the simpler approach

Use of Elaborate Graduation Formulas and Curve Fitting In the special case where $k = 2$ in relations [16] and [17] we have the equivalent of application of simple moving averages for $K = 2$ to like moving averages in [3], i e , a double graduation This and many other forms of weighted moving average as well as other graduation formulas (29a) might be used advantageously in some situations Indeed, methods that involve fitting of curves of given type (3–20, 30) may be regarded as elaborations of such methods, useful to replace a basic method such as that now proposed, where there is good reason to expect use of the more elaborate method to lead (without introduction of intolerable bias) to important improvement in precision or to permissible abbreviation of tests (3–12) Of course, other percentiles may be used as "endpoint" instead of the median, but usually less efficiently However, it may not be readily apparent what type of curves should be useful Wilson and Worcester have thus been led to consider (30) a generalization of the curve fitting problem in bioassay

The investigations by Hussey and associates (31–35) of effects of radiations on biological systems furnish an illustration (5a), both for quantal and gradational assay, of strange characteristics that may be encountered in a dosage-response curve Furthermore, these studies provide an interesting example of a mistaken attempt (by another writer) to approximate the curve involved by fitting a fairly simple increasing function of the irradiation interval (t) to the average duration of the prepupal stage (ϕ), employing data of the first report This is discussed in one of the later papers (33), further exploration having clearly demonstrated that such dosage-response curves on the contrary rise to a maximum value of ϕ and then fall to an almost level plateau in all cases investigated (32, 33) In the later article (33) the median is used as average in preference (5a, 13, 36) to the mean, and in the second text-figure (reproduced elsewhere (5a)) one experience is illustrated by a combined graph of median response ϕ and a class-frequency diagram of individual responses corresponding to each irradiation period t that was employed A *quantal* assay system could be imagined as based on responses found up to any convenient value of ϕ (the abscissa of the graph), but it is easy to see how an unfortunate choice, say $\phi = 7.5$ days, would lead to ambiguities in tests and insensitivity with change in dosage above 160 minutes under the given conditions

That difficulties of this sort may be encountered in immunologic assay is illustrated in the studies of mouse protection tests by Goodner and Horsfall (37) With certain pneumococcus antisera given to test animals in varying amounts to counter a standard dose of pneumococcus culture (sufficient to kill practically all animals otherwise) they found that over part of the range increased protection appeared to result from increased antiserum dosage, but with a further increased dosage a maximum protective effect apparently was passed, for beyond a certain point increased dosage appeared to give less protection If such a system must be used in assay, then care must be taken to choose conditions so as to deal with the required branch of the fundamental curve or change conditions so as to eliminate the peculiarity

An interesting case in point is presented by some of the complement-fixation systems We have indicated that the amount of complement M required for 50 per cent hemolysis is a linear function of the amount of a given antiserum used in a reaction with the essentially *optimal amount* of antigen With some systems (e g , the tuberculosis tests) it is enough that the amount of antigen be in considerable excess, but with others (e g , tests for syphilis or pneumococcus antibody) there is an amount of antigen that gives a maximal fixation

reaction with the given amount of antiserum and less fixation is obtained with either more or less antigen Fortunately, in the syphilitic system the curve does not have a sharply defined maximum but a fairly broad range in which nearly maximal effects are obtained Still more fortunately, the maximally effective amount of antigen depends essentially on the required maximal M, which in turn is approximately dependent on the amount of antibody present, and not capriciously on the particular qualities of the serum otherwise Accordingly we may set up (as in the routine test for syphilis) three tubes containing respectively 3, 6, and 12 units of complement and in each the amounts of antigen appropriate if the value of M falls within range of estimation from the resulting p found

Features of Weighting Systems in Curve Fitting At this point it seems appropriate to examine at least the approximate influence of weighting systems commonly employed in the curve-fitting process, but in approaching this subject, perhaps like Berkson (19), we should pray for guidance At least we may hope that the discussion will serve as a stimulant to others As previously, we take $\log D$ as abscissa in both the fundamental and transformed coordinate systems Accordingly, let w be the weight assigned to the squared vertical deviation of a point (observed or "corrected") from the fitted line, and assume we are to minimize the sum of the weighted squares in the fitting process

The $T_{(p)}$ transformation is equivalent to a one-way stretching of the coordinate plane vertically in either direction from the $p = 0.5$ horizontal The factor for the total *stretching* from the transformation of a point not on that horizontal is $[T_{(p)} - T_{(0.5)}]/(p - 0.5)$ As previously, it is convenient but not necessary to have $T_{(p)}$ an increasing function of p, then this total-stretch factor is positive However, the local distortion or *stretching factor*, which we shall denote by $1/E$, is given by the derivative of $T_{(p)}$ with respect to p Thus, with base e logarithms if $T_{(p)} = \log \dfrac{p}{1 - p}$, then $E = pq$, or if $T_{(p)} =$ either the normal deviate or the probit of p, then E is the corresponding ordinate (z) of the *normal* curve Use of logarithms to any other base (say a) instead with $T_{(p)} = \log \dfrac{p}{1 - p}$ would give $E = pq \log_a e$, a constant times the value $E = pq$ for natural logarithms Of course, any set of weighting coefficients may be multiplied by any constant throughout without essential alteration in the result of the curve fitting

Now, let w' represent a weight we might agree to give to squared deviations in p from the fundamental ($\log D$, p) curve approximation, e g., w' might be taken as the reciprocal of the estimated variance of p in random sampling, then $w' = n/(pq)$ Hence we might expect $w = E^2w'$, and approximately this is the weighting system used by Bliss (14) and Berkson (19, 20) with the appropriate interpretation of E, although adjustment of the transformed data may be involved also

Obviously, the corresponding negative transformation, $-T_{(p)}$, gives the same weighting coefficient $w = E^2w'$ Thus for the logit transformation the weighting coefficients may be taken as $w \cong np^2q^2/(pq) = npq$ It is true here and also, but less markedly, with the probit system that less weight is given to points in the *transformed* coordinates the further p is from 0 5, but we should guard against the fallacy of supposing that therefore original points ($\log D$, p) are given less

weight according as p is remote from 0.5. Indeed, the opposite is clearly evident from the derivation as Garwood (16) and Berkson (20) have emphasized.

As has been noted, the difficulty with $p = 0$ or $p = 1$ in the observations has been treated (14, 15, 20) by adjustment of data. Strictly in accord with the principle applied, all points should be adjusted (14). The adjustment is based on a straight line fitted by inspection or otherwise to the transformed points or successive approximations by straight lines generated from that beginning. The "corrected" or "fictitious" points employed by Bliss (14) and Fisher (15) are taken so that the weighted sum of squared deviations from the provisional line is $\sum [n_i(p_i - p_i')^2/(p_i'q_i')]$, where p_i' is the corresponding p-value on the provisional line and $q_i' = 1 - p_i'$, and the process is aimed at finding a line which makes this sum a minimum, and is essentially equivalent to fitting an integrated normal curve (16) to the points in the fundamental form so as to minimize this sum. The logistic function instead of the integrated normal curve may be employed in a similar manner (17, 18, 20).

It is at least of theoretical importance to note that, apparently, the conditions for convergence of the curve-fitting process have not been carefully specified. Good examples have been given, especially by Garwood (16), to suggest the nature of the processes involved, but a general theorem of convergence has not been proved for any set of points $(\log D_i, p_i)$, or with certain specified exceptions, where we are required to fit a given curve or a straight line in transformed coordinates in the indicated manner. Indeed, the general theorem without such exceptions can easily be disproved, and for this it is sufficient to cite a single example of failure. Thus for any set of doses, $D_1 < D_2 < D_3$ or more specifically $D_3 = 2D_2 = 4D_1$, let the values of p_i be given by $p_1 = 0 < p_2 < 1$, and $p_3 = 1$. In this example the method of Bliss and Fisher would not direct us toward any line of finite slope and the estimated median-effective dose would appear to be D_2. A second example with just the first two points instead would yield essentially the same result, the estimate of M being D_2. In either case, this would be so regardless of the value of p_2.

The present purpose is not to determine the limitations of these methods, but to indicate that the issue should be investigated at least to the extent of specification of some definite *practical limitations*. Of course, no method of estimation of median-effective dose should be applied to data where p_i is constant in the given experience (49), nor if for a given i we have $p_{i+j} = p_{i-j}$ throughout the data utilized, nor without restriction of range to exclude the influence of a possible indefinite extension of dosage values with the *true* $\bar{p}_i$ approaching asymptotically some value other than zero or one. However, the examples cited above are not of that type and demonstrate that other exceptions are required. An immediately suggested expedient might be based on the fairly common practice of excluding all values of p_i that are zero or one in obtaining the first provisional line and requiring thus that at least two other values of p_i remain. Then we might proceed to obtain the second provisional line with exclusion of all points for which the *expected value* p_i' (taken from the preceding line) is not in the interval (0.001, 0.999), and to obtain any successive fitted lines with exclusion of points for which p_i' on the immediately preceding line is outside (0.05, 0.95) or some other preassigned interval. Obviously, at least two points must remain to yield the line. Such a system might overcome some of the indicated difficulties, and appears to offer only an objective direction of what might be expected to result from good judgment.

Another difficulty, not apparent in the mathematics based upon the original assumptions, arises as a result of infringement of one of the assumptions, made at least tacitly, that other sources of variation in the observations $(\log D, p)$ are negligible with respect to the variation in p from random sampling. Perhaps we might stretch the condition to

include cases where the other sources of variation are no more influential (48) However, as Neyman (12c) has pointed out, this is not necessarily so Indeed, in any practical situation, this assumption implies that n is not so great as to make the variance of p negligible with respect to contributing variance arising from other sources, and, as contrary cases, we have previously cited the complement-fixation systems where $n \geqq 10^8$ and thus $2\sqrt{pq/n} \leqq 0\ 0001$ Such conditions effectively undermine the basis on which there has been established a preference for taking deviations in the given direction This is not enough to establish the contrary preference, but certainly throws the question open again, and in such cases invalidates the weighting system described above

Still another difficulty lies in the use of the *index of dispersion*, $\Sigma[n_i(p_i - p'_i)^2/(p'_i q'_i)]$, as the basis for approximating a *maximum likelihood* fit It is well known (27, 28) that, especially for small values of n_i and for p'_i remote from 0 5, the approximations involved may be poor Accordingly, with any given data any method so based, such as that of Bliss and Fisher (14–16), should not be considered even to *aim* at an *exact* maximum likelihood solution, and reference to it as "the exact" method (23) even in quotation marks may lead to a false appreciation However, this deflection of aim does not appear to detract from the practical excellence of these curve-fitting methods when suitably applied

Moving-Average Interpolation Viewed as a Basic Method In certain situations where it may seem desirable to compare the efficiency of a given method of estimating the median-effective dose M with that of a *basic* method of simple character, the simple moving-average interpolation may well serve the purpose It possesses the most desirable basic characteristics independence of assumption about the precise form of the *fundamental* (logarithm of dosage, relative response frequency) curve, and use of well-known principles of graduation and interpolation in the estimation As the necessary calculations are simple, it may be regarded as the method of choice unless another is shown considerably more efficient in a given situation A choice has to be made here, as elsewhere (36a), between methods which seem to rest on a relatively secure base and others which involve an apparent risk in further assumption but offer a prospect of gain in either power or economy or both Comparison of a more aggressive method with a method more *basic* in the given sense may seem entirely unnecessary if other evidence of value may be obtainable (11, 12, 44, 45) as has been mentioned in the discussion of complement-fixation tests Caution is desired but *not* timidity

Of course, the fundamental curve should be approximately symmetrical with respect to the *median-point* (log M, 0 5) at least within the interval (log D_a, log D_b) to be well-suited to estimation of that point by the linear interpolation from simple moving averages However, by suitable limitation of range (made possible by choice of d, n, and dosage range in the test plan and use of an appropriate value of K) good conditions for estimation of M in this way should be attainable in almost any situation where the notion of median-effective dose is useful Indeed, its definition is based essentially on the supposed possibility of its estimation by the method of simple interpolation, equivalent to the special case $K = 1$ in formal use of relation [6] The types of curves (logistic and integrated normal) most used as approximations of the *fundamental curve form* are S-shaped symmetrically about the estimated median-point (log M', 0 5), thus even greater emphasis is placed on a condition that is favorable to use of

the moving-average method. Furthermore, as Berkson (20) has pointed out and we have indicated above, both his own method and that of Bliss and Fisher operate approximately as if to fit a curve (respectively, the logistic and the integrated normal) to the data in the fundamental form of points ($\log D_i$, p_i) with a *weight* proportional to $n_i/p'_i q'_i$ assigned to squared deviations, $(p_i - p'_i)^2$, where p'_i is the ordinate of the point ($\log D_i$, p'_i) *on the fitted curve*. Thus, these methods give relatively more weight to the original ($\log D$, p) points according as they are more remote from the estimated median-point ($\log M'$, 0.5). The simple moving-average interpolation method does not do this. Equal weight is given to all points utilized in the actual calculation except the first and last, which are given weights no greater—usually somewhat less—automatically dependent on the value of f as may be seen in the Appendix, and all other points have no weight except in the preliminary choice of the range (a, b) to be used for the index i in the interpolation. This feature of the proposed basic method seems at worst to err on the side of safety. It should tend to make comparative tests sensitive to any increase in efficiency resulting from use of a curve-fitting method that places special emphasis upon the data of points remote from the median-point.

Finally, the writer wishes to make it very clear that he has no intention of discouraging use of the curve-fitting methods, which appear to have found many valuable applications.

APPENDIX

ESTIMATES OF ASSAY VARIANCE, BASED ON INTRACLASS DATA

We have noted under relation [6] that as long as f lies in the *unit interval* the same value of a is suitable for the required interpolation, i.e., an increment (Δf) in f produces d times as great an increment in $\log m$, $\Delta \log m = d\ \Delta f$, where d is a constant as previously defined.

Furthermore, the partial derivative of f with respect to p_i is readily obtained for any i. From relation [6], for $p_i = r_i/n_i$, we have

[A1]
$$f = \left(\frac{K}{2} - p_a - \cdots - p_{b-1}\right) \Big/ (p_b - p_a), \quad \text{and}$$
$$f - 1 = \left(\frac{K}{2} - p_{a+1} - \cdots - p_b\right) \Big/ (p_b - p_a)$$

Therefore, the partial derivatives are

[A2]
$$\frac{\partial f}{\partial p_i} = \frac{-1}{p_b - p_a} \qquad \text{for } a < i < b, \text{ and}$$
$$\frac{\partial f}{\partial p_a} = \frac{\partial (f-1)}{\partial p_a} = \frac{\frac{K}{2} - p_{a+1} - \cdots - p_b}{(p_b - p_a)^2} = \frac{f-1}{p_b - p_a}, \text{ and}$$
$$\frac{\partial f}{\partial p_b} = \frac{\frac{K}{2} - p_a - \cdots - p_{b-1}}{-(p_b - p_a)^2} = \frac{-f}{p_b - p_a}$$

Let $\Delta p_i = p_i - \bar{p}_i$, then Δf may be approximated by

$$\text{[A3]}\quad \Delta f \cong \frac{-1}{\bar{p}_b - \bar{p}_a}\ [(1 - \bar{f})\ \ \Delta p_a + \Delta p_{a+1} + \quad + \Delta p_{b-1} + \bar{f}\ \ \Delta p_b],$$

where $\bar{f}$ is the value of f for the case where p_i is replaced by $\bar{p}_i$ throughout formula [A1] Obviously, the *expected value* of Δf is approximately zero, $\overline{\Delta f} \cong 0$, whence the expected value of $(\Delta f)^2$ is approximately the variance of f, $\overline{(\Delta f)^2} \cong \sigma_f^2$ Assume that Δp_i is independent of Δp_j for $i \neq j$ Then the expected value of the cross product is given by

$$\text{[A4]}\quad \overline{(\Delta p_i)(\Delta p_j)} = 0 \text{ for } i \neq j$$
$$= \sigma_{p_i}^2 \text{ for } i = j$$

Accordingly, the variance of f may be estimated by

$$\text{[A5]}\quad \sigma_f^2 \cong \frac{1}{(\bar{p}_b - \bar{p}_a)^2}\quad [(1 - \bar{f})^2\ \ \sigma_{p_a}^2 + \sigma_{p_{a+1}}^2 + \quad + \sigma_{p_{b-1}}^2 + \bar{f}^2\ \ \sigma_{p_b}^2]$$

By definition, $s_i = n_i - r_i$ and $q_i = s_i/n_i = 1 - p_i$, and, accordingly, $\bar{q}_i = 1 - \bar{p}_i$ The *true* variance of p_i is $\sigma_{p_i}^2 = \bar{p}_i\ \bar{q}_i/n_i$ as is well known, and this variance may be estimated from the sample value (p_i), for $n_i > 1$, by

$$\text{[A6]}\quad v_i = p_i\ q_i/(n_i - 1)$$

This is also well known in a more general form, but is readily verified in this special case (*temporarily* dropping the index i) from relations based on the point binomial, as follows

$$n^2(n - 1)\quad \bar{v} = \sum_{\alpha=0}^{n} \binom{n}{\alpha}\ \ \bar{p}^{\alpha}\ \ \bar{q}^{\beta}\ \ \alpha\beta$$

[A7] where $\beta = n - \alpha$, whence, for $n > 1$,

$$n\quad \bar{v} = \sum_{\alpha=1}^{n-1} \binom{n-2}{\alpha-1}\ \ \bar{p}^{\alpha-1}\ \ \bar{q}^{\beta-1}\ \ (\bar{p}\bar{q}) = \bar{p}\ \ \bar{q}$$

Accordingly, the expected value of v_i is $\bar{v}_i = \bar{p}_i\ \bar{q}_i/n_i = \sigma_{p_i}^2$, the *true* variance of p_i Thus, as the variance of log m is approximately d^2 times that of f, we obtain from [A5] and [A6] the estimation formula,

$$\text{[A8]}\quad \sigma_{\log m}^2 \cong \frac{d^2}{(\bar{p}_b - \bar{p}_a)^2}\quad [(1 - \bar{f})^2\ \ v_a + v_{a+1} + \quad + v_{b-1} + \bar{f}^2\ \ v_b],$$

wherein, unfortunately, there still remain three quantities ($\bar{p}_b$, $\bar{p}_a$, and $\bar{f}$) that are not given by the assay experience, except that they may be approximated by the corresponding sample values (p_b, p_a, and f) However, the additional error in approximation thus induced may be so small as to be negligible, if $\bar{p}_b \cong 1$ and $\bar{p}_a \cong 0$, in comparison with other sources of error in the approximation [A8] Estimates of variance obtained in this way are not adapted to such refined tests of significance (27, 28) as is the variance estimate s'^2 defined *in the text in accord with Rider's (28) notation* Of course, if replicate assays have been made under essentially the same conditions, then composite estimates may be made or data may be pooled under some circumstances for the purposes

In the case of constant $n_i = n > 1$, relation [A8] may be reduced to

$$\text{[A9]}\quad \sigma_{\log m} \cong \frac{d}{r_b - r_a}\ \sqrt{\frac{(1 - f)^2\ \ r_a s_a + r_{a+1}s_{a+1} + \quad + r_{b-1}s_{b-1} + f^2\ \ r_b s_b}{n - 1}}$$

where, of course, the standard deviation estimate is positive or zero, the same value is given in this case ($n_i = n$) with r_i replaced by p_i and s_i by q_i throughout

However, in a consideration of possible test-plan improvements, with regard to the influence of choice of values for n, d, and the moving-average index span ($K = b - a$), it is convenient to have in mind the form derived by substitution of the hypothetical true variance, $\bar{p}_i\bar{q}_i/n$, for $\sigma^2_{p_i}$ in relation [A5]

$$[A10]\quad \sigma_{\log m} \cong \frac{d}{\bar{p}_b - \bar{p}_a} \sqrt{\frac{(1-f)^2 \quad \bar{p}_a\bar{q}_a + \bar{p}_{a+1}\bar{q}_{a+1} + \quad\quad + \bar{p}_{b-1}\bar{q}_{b-1} + f^2 \quad \bar{p}_b\bar{q}_b}{n}}$$

All the indicated approximations are asymptotic as $n_i \to \infty$, for $i = a$, , b As indicated in the main text, these formulas are applicable only where unrestricted random sampling is employed or where an estimate of relative efficiency in use of other sampling technics is to be made in default of control experience with unrestricted random sampling In the latter case direct estimates (s'^2) of variance of log m from replicate assays with the given sampling technic are required for the comparison, they are desirable in any case The indirect estimates obviously are insensitive to technical errors such as those arising in use of a syringe, or in failure to have comparable environmental conditions at the time of administration of the test dose to subjects and thereafter to the end of the observation interval Many such influences are likely to be greater when assays are not performed simultaneously Nevertheless, non-simultaneous replicate assays may be preferred in order to avoid too great reliance on results that may be abnormally affected by a temporary condition of the colony of test subjects An example might be given by replicate assays where two reagent solutions are tested for relative potency, ostensibly of a given agent There may be other materials present in different relative amount in the two solutions and sensitivity of test animals (subjects) may differ from time to time, not only with respect to the agent in question but with respect to the other materials also This would be an unfortunate situation, of course, but to remain ignorant of it would be more unfortunate Accordingly, non-simultaneous replicates may seem preferable if such influences are suspected Likewise, it seems usually preferable to compare reagent preparations with a standard (4, 5, 11, 12) agent rather than accept the reactions of a colony of subjects as a standard

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EDITOR
BARNETT COHEN

It is characteristic of Science and Progress that they continually open new fields to our vision—PASTEUR

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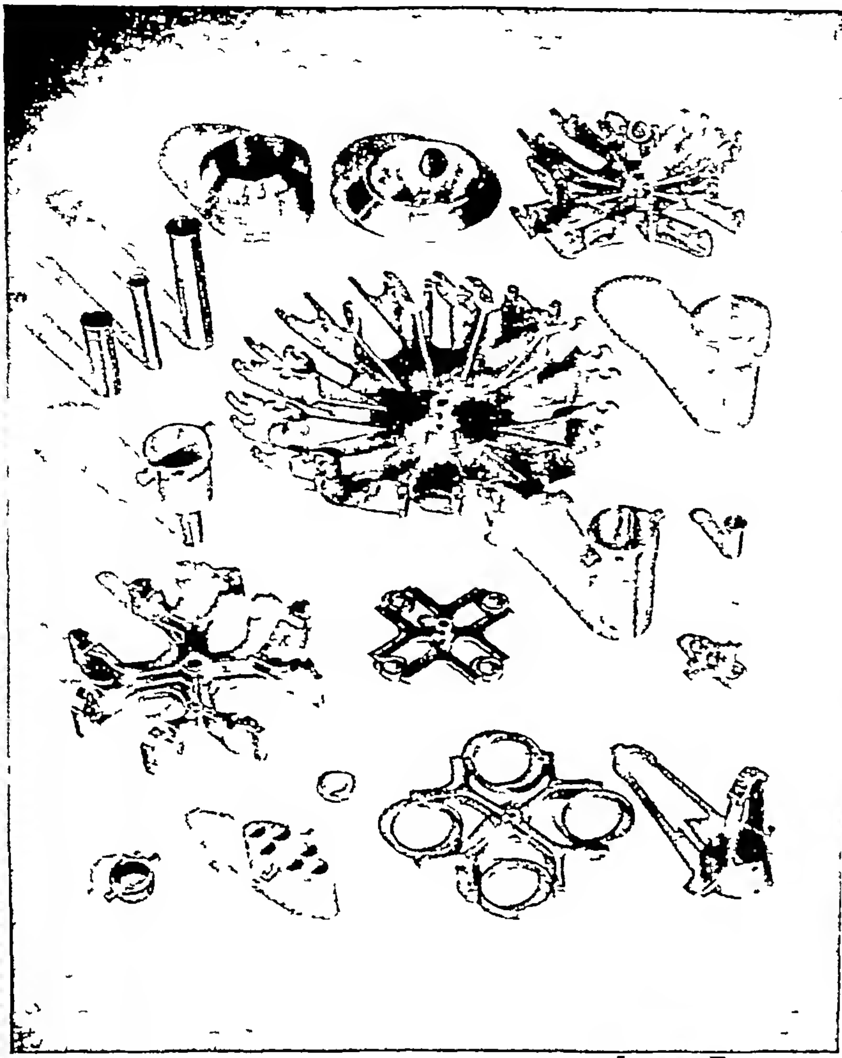

MECHANISMS OF INFECTION AND IMMUNITY IN VIRUS DISEASES OF MAN[1]

THOMAS FRANCIS, JR

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The biologist concerned with the microbic world has numerous approaches with which to gain pertinent information of the agents with which he is concerned In many instances attention is centered upon morphologic and basic composition of the species studied, but in a great proportion the detection of specific properties and their evaluation are based on measurements of the behavior of the agent upon specific substrates When a living organism is the major site of recognized activity of the parasite it becomes the substrate, and if the action of the parasite creates injury and active response on the part of the host the reaction constitutes disease Theoretically, it should be possible to measure the specific components of host and parasite which are involved in a primary injury but the instances in which it has been accomplished are extremely few And even where special activities of the parasite have been found to be exerted upon definite tissues the manner in which the effects are brought about is in most instances obscure—limited largely to a description of the morphologic changes in cells which the agent attacks or to the type of inflammatory response which follows The fundamental interactions which determine selective localization and pathologic injury are not known Conversely, the phenomena which differentiate naturally susceptible tissues and species from the resistant are essentially unknown

Nevertheless, the ability of the susceptible host to modify his behavior after proper experience with a given agent has been seen to be associated with the acquisition or orientation of mechanisms not commonly encountered in the naturally resistant host It has been increasingly obvious that regardless of the biologic level of the parasite, multicellular, bacterial or viral, the responses of the animal host are much the same, due in part perhaps to limitations in experimental methods of measuring them In accordance with this trend a number of features formerly said to differentiate reactions to viruses from those to other infectious agents have fallen away or have required sharp limitations They include the concepts that permanent immunity is uniform, that antibodies can be elicited only in a fully susceptible animal, that serological reactions such as precipitation, complement-fixation and agglutination are essentially directed against components of infected tissues of the host rather than to the virus moiety, that immunity can be obtained only by active infection

An effort to bring the reactions of immunity to virus infections into conformity with those of other disease-producing parasites may appear on the one hand to be

[1] Presidential Address delivered before the Society of American Bacteriologists at its Forty-seventh General Meeting, Philadelphia, Pa , May 14, 1947

ultra-conservative as seeking to limit philosophic exploration, or progressive in seeking to apply a general law to presumably dissimilar behaviors Many of the ideas currently debated in the virus field are direct transfers from those of bacterial and other parasitic infections cellular immunity independent of antibodies, immunity and antibodies derived only by persistence of the parasite, the significance of strain variation, the advantage of infection over inactivated organisms in producing immunity, the meaning of antibodies in terms of antigenic structure, the inefficacy of specific serum therapy In the latter connection it is important to recall how few are the instances, to date, of effective serum treatment of bacterial diseases or of effective prophylactic immunization apart from those involving circulating exotoxins One can here point out as well that as procedures for studying the various diseases have improved the role of independent tissue immunity has declined, leaving it a prominent factor in only those diseases for which serological procedures are undeveloped, and that an understanding of the participation of antibodies in terms of bacterial constitution has scarcely been broached

The patterns followed by viruses in creating disease are as different and complex, or more so, as those observed with other classes of parasites, although the viruses represent a more homogeneous biological group in that they are considered obligate intracellular parasites Nevertheless, the routes of infection, the tissues attacked, and the character of injury induced vary so widely it seems impossible to consider the mechanisms of immunity except in terms of the mechanisms of infection, i e, the pathogenesis of the different diseases Despite the inadequacies in precise data there are many clinical and epidemiological observations of the virus diseases of man which, combined with immunological studies, afford opportunities to visualize the mode of action of viruses as reflected in the responses of the host Conversely, an understanding of the mechanisms of infection at play offers a much better opportunity to clarify the natural history of a disease and the direction of specific control measures The discussion will deal primarily with virus diseases of man and the significance of protective antibodies when considered in terms of the disease process, the portal of entry, the type of tissue injury, and the fate of the virus But the opportunity will be taken to comment on other characteristics of viruses suggested by the study of the disease processes Discussion will be largely limited to virus diseases of man Those of plants will be carefully avoided for while they offer many interesting aspects, the reported absence of antibodies, the transformation of a high proportion of the plant proteins to virus, the limited conditions under which infection can be instituted, and other features suggest at present red herrings rather than aids in interpreting diseases of man

Similarities in disease pattern, pathogenesis, and the agents involved tend readily to separate certain of the virus diseases of man into groups which illustrate the sharp differences in immunity exhibited, and also invite explanations of the common and discrepant features of the constituent groups

Four sets of conditions suggest themselves *1*, virus diseases in which infection persists but immunity does not, *2*, virus diseases in which immunity persists but

infection does not, *3*, virus diseases in which neither infection nor immunity persists, *4*, virus diseases in which both virus infection and immunity persist In order to fit some of the examples into the groups suggested, a certain amount of freedom has been taken with established concepts and the classifications may have been stretched a bit, but with the hope of provocation rather than of pure distortion

Group 1 Virus Diseases in which Virus Infection Persists but Immunity does not The agents of the Psittacosis-Lymphogranuloma venereum group clearly belong in this category In their natural hosts, especially, they may be initially introduced and maintained as resident or carrier infections with little general evidence other than serological reaction to indicate that the virus has been present Under these conditions the virus is contained by the host in a well adjusted stage of parasitism But exposed to physiologic insult which may disturb the equilibrium, the host factor is depressed and the virus may assume the offensive, reappearing in organs and their excreta so as to make the animal an active distributor

In man the details are less clearly established Nevertheless, with lymphogranuloma venereum there are the recorded evidence of prostitutes transmitting the disease without significant signs of its presence, the known reservoir among the colored population especially in warm climates, and the continued chronic infection which can occur after chemotherapy In psittacosis *of man* several instances have been recorded which Meyer has suggested may be actual relapses rather than newly acquired second attacks, and certain individuals have given strong evidence of being carriers

The fact that other agents of the group follow so distinct a pattern in their native hosts is strong indication to support the available data in man The viruses of pneumonitis in mice, hamsters, cats, and ferrets have all been encountered in stock being subjected to inoculations attempting to establish other viruses, thus clearly demonstrating the activation of a carrier state

One of the theses proposed in explanation of continued immunity ascribes that fact to the persistence of virus in the host's tissues The same thesis also attributes the persistence of protective antibodies for a prolonged period after infection to the continuing stimulus of virus remaining in the tissues Diseases associated with the group of viruses mentioned are characterized by persistence of virus which tends to maintain itself through the proliferative and exudative response in lymphatic tissue and serous membranes Rather than exhibiting a permanent immunity, however, they are typically subject to relapse They represent persistent, unstable but balanced infections instead of high grade immunities The failure to respond to inoculation of the same agent with a chain of reactions similar to that exhibited by the uninfected host is commonly called infection immunity It is scarcely immunity but a stage of tolerance to amounts of infectious agent which appear insignificant in terms of the amount available in the tissues of the infected host

The diseases of this group strikingly exemplify the equilibrium between infection and resistance, between host and parasite Moreover, if these be invasions

by the host species' indigenous parasites they illustrate how troublesome a problem infection with one's own saprophytes may be Viruses of the group have also been found to be susceptible to sulfonamides and penicillin, leading to the suggestion that an extra-cellular phase of metabolism is an essential stage in their cycle It has also been suggested that these agents do not represent the true viruses, but it is difficult to see why they should be separated from a class of agents so inhomogeneous and poorly defined Certainly, they belong as much as the pox viruses do, and one can speculate that because of their similarities the poxes may be the next ones to respond to drugs and antibiotics

Infection with the virus of lymphocytic choriomeningitis in mice, dogs, monkeys, and probably man displays similar unstable continued infection even when antibodies are present in good amounts

Special care has been taken to avoid the problem of the cold sore, herpes febrilis The evidence at present available is generally interpreted to indicate that the initial experience with that virus produces a diffuse infection and a general immunity with circulating antibodies There is little information of the persistence of virus deep in the tissues, but several reports describe the residence of virus in the mouth and its appendages The current concept is, then, that herpes virus persists in superficial tissues and erupts when insult to the area occurs Furthermore, the individuals in which virus recurs possess good levels of circulating antibody The picture strongly suggests an accelerated local reaction From the point of view of pathogenetic pattern, I would be much happier if the recurrent attacks of fever blisters could be shown to be newly introduced infections, for in other ways this virus has close similarities to those assigned to the third group and I shall ask the privilege of taking up certain points at that time even if it be a little out of keeping with usual interpretations

Group 2 Virus Diseases in which Immunity Persists and Evidence Indicates that Virus does not A Infections limited to man The rash-producing infections, the exanthemata, of virus etiology appear historically and epidemiologically to be diseases which in their evolution have become so thoroughly adapted to man as to have eliminated other hosts from their infection cycle Smallpox, measles, chickenpox, are characterized by high transmissibility by the respiratory route, an incubation period of ten to twenty days, a period of fever, catarrhal symptoms, and blood invasion followed by florid cutaneous localization Second attacks of the full blown disease do not occur so that immunity appears to be a permanent result of the primary attack. Relapses are not noted Nor do segregated populations which include a nucleus of previous cases of disease appear to derive infection or immunity from association with those individuals who should be the carriers of virus Infection is introduced by the fresh case Here, then, are instances of virus disease which are maintained apparently by the human species alone, which elicit prolonged immunity, which result in the production of antibodies for many years, but in which evidence of continued residence of the virus has never been presented Interpretation of the pathogenetic mechanisms offer, however, a reasonable explanation of why this group of diseases yield lasting immunity

The portal of entry and the primary site of localization of virus appear to be in the upper respiratory tract from which the virus progressively extends. With the onset of acute illness invasion of the blood stream takes place and, along with lesions of the mucous membranes, a secondary localization of the virus occurs in the skin producing the characteristic rash upon which clinical identification largely depends. After recovery, a rich supply of circulating antibodies is provided and if virus were again to be introduced it might be able to establish itself superficially in the mucous membranes, but the stage of extension through the blood to produce the diagnostic eruption would be eliminated by the circulating antibodies which the virus would encounter. Under these conditions it is not unreasonable to believe that repeated upper respiratory infections with measles, smallpox or chickenpox viruses can occur and that the presumed permanent immunity is, in fact, immunity only against the generalized disease.

That measles can actually occur a second time is seen under two conditions. Rake, Stokes, Shaffer and their associates, cultivated in eggs what appeared to be measles virus as demonstrated by its ability to elicit a mild disease with Koplik spots and rash. Among infants tested for immunity to unmodified measles within a few months after the modified disease, clinical measles again occurred, indicating that infection with an active virus had not resulted in uniform immunity.

It is extremely interesting in terms of pathogenesis of the disease to observe the gradations from absence of disease to but slight modification of measles which can be attained by use of antibody after exposure to infection. The fact that complete protection is readily gained within three days after exposure suggests that either the virus has not entered vulnerable cells by that time or that the number of cells parasitized is too small to yield a significant amount of virus for transportation beyond the portal of entry. The modification of disease which takes place when serum is not given until the sixth day suggests that virus has been sufficiently distributed so that great amounts of antibody are required to catch all that has been produced. The modified disease is, however, measles infection and pediatricians assure me that children having had the modified disease can contract measles again after a later exposure, demonstrating that mild, active disease has not given permanent immunity.

Moreover, vaccine virus may be attenuated to such a degree that while a primary lesion results from its inoculation it does not give more than a brief protection to the more virulent parent lymph-strain. Even with a potent vaccine strain, *immunity* is not *permanent although* vaccination represents *frank* infection with active virus. The modified strains might well be considered to represent stages of parasitism which could or should become resident and persist, but do not. These observations also indicate that induced variation and attenuation must be of a limited nature so as to retain the proper immunogenic components in order to function protectively.

One wonders whether these diseases, with their fixed patterns and prolonged incubation periods in man, have not come to depend upon their presence in the

human host to complete a definite phase in their life cycles, thus accounting for their uniform, predictable behavior, and whether the persistent immunity is related to a genetic conditioning of the human cell—acquired through long experience with the same agent—which results in ready production of immune substances Certainly, there is no reason to believe that secondary exposures and minor infections would not participate as secondary stimuli of the flush mechanism or booster effect

B Infections transmitted by biting insects This group of diseases is derived from other hosts and transmitted to man by insect vectors which introduce the virus into the blood as feeding occurs The site of essential injury is a distant organ to which the agent is transported by way of the blood stream and there localized to multiply and produce the characteristic disease Yellow fever is a splendid example as is the group of viruses associated with acute encephalitis which appear to be variants of one basic agent In one instance, the liver serves as the major site of injury, in the other, the brain Mosquitoes are the most frequent vectors and apparently bring the virus to the human individual in a vegetative stage readily adaptable to human tissues, since prolonged incubation periods are not required Immunity develops after infection and is persistent, that from yellow fever is essentially permanent It is easy to see why Antibodies are present in the blood so that when virus is again inoculated by the blood sucking insect, the virus introduced into the blood is immediately met by antibody and neutralized There is little evidence of persistence of virus in man (the virus of St Louis encephalitis persists for a time in experimental animals), relapses of infection are not described, and artificial immunization can be effectively done The conditions are ideal for circulating antibodies to be most efficient Other diseases presenting similar mechanisms of infection should behave in a similar fashion Three years ago I ventured to predict, because of the disease pattern, that if dengue did not result in permanent immunity it probably indicated the existence of antigenically different strains, a fact since demonstrated by the beautiful studies of Sabin

It is of further interest to point out that inactive virus can induce immunity to Japanese B encephalitis and equine encephalomyelitis, whereas the antibody titer resulting from vaccination with active yellow fever virus gradually declines although it represents the response to an active infection

Group 3 Virus Diseases in which neither Virus Infection nor Immunity Persists In contrast to those virus diseases of man in which evidence points to an effective, prolonged immunity there are certain others in which evidence of repeated infection is clear Among them are influenza, common cold, foot-and-mouth disease, possibly poliomyelitis, and herpes, maybe Although they appear at first glance to have little in common, they are characterized by short incubation periods and by the fact that the primary injury is to a superficial tissue

Regardless of whether herpes virus is aroused from the tissues or freshly introduced, the recurrent infection is limited to a local lesion in the superficial squamous epithelium The virus can become established and institute injury in

surface cells not readily reached by antibodies in the circulating blood which, nevertheless, can and *do* limit extension beyond the local site Recently Evans, Slavin, and Berry have demonstrated that induced passive immunity can, in the experimental animal, similarly restrict infection with herpes virus to a local lesion, results duplicating those obtained with foot-and-mouth disease by earlier investigators In fact, the pathogenesis of recurrent local infection in foot-and-mouth disease bears close resemblance to that presented for herpes

Influenza virus selectively destroys the ciliated epithelium of the respiratory tract In this instance, again, the susceptible cells to which the virus is readily introduced through inspired air, are superficial and essentially extravascular Repeated infection with the same virus can occur in man or animal even though antibodies are present in the blood, and the epithelium is more than once a site of injury But the damage is limited to a local lesion and the extension of injury to characteristic pulmonary disease is prevented It has been clearly shown in influenza that the secretions of the respiratory tract may contain antibody which is enhanced in amount after infection or vaccination, paralleling at a lower level the content of antibodies in the blood, and it seems probable that the antibody exuding into the local secretions are the significant factors in determining whether virus can become established while the circulating antibody itself bountifully supplies and protects the lungs The protective effect of vaccination by para-respiratory route can be attributed to its influence upon the antibody content of the respiratory secretions On the other hand, repeated infections may, in addition, result in functional modifications of the epithelium which make it less likely to virus injury Persistent virus in man has not been demonstrated In swine, Shope has presented evidence that virus in the form of masked marvels can be maintained in worm parasites in the hog's lung,—*but* it does not give rise to immunity, and disturbance of that equilibrium incites disease

Although influenza virus behaves as a perfectly good member of that class of agents, certain features are intriguing enough to stimulate the question as to whether it is of necessity an obligate intracellular parasite The speed of its action is such that clinical disease occurs and cell destruction takes place in as little as twenty-four hours In vaccinated animals the process may even be accelerated but virus is difficult to detect, thus suggesting its superficial neutralization The manner in which influenza virus attaches to red blood cells and elutes is a striking example of virus attachment without apparent penetration Moreover, Hirst has shown that the same phenomenon occurs when the virus is brought in contact with respiratory tissues of the ferret or mouse Virus adsorbed to red cells is apparently still neutralizable by antibody The comparative inefficacy of repeated dosage of virus in heightening antibody titer suggests that a surface antigen is of primary significance If virus be given to mice by the intraperitoneal or intravenous route, it ordinarily does not cause typical pulmonary lesions although plenty of virus can be found in suspensions of lung tissue One might anticipate that the virus would be able to penetrate the desired cells from the other side of the membrane, but lesions seem to occur

only when very large amounts are given In that case the virus appears to overflow from the blood into the upper respiratory tract where it takes up its pathogenic action on the surface of the epithelium Supporting this concept, experiments, as yet unpublished, have shown that a small amount of serum given into the nose protects against pulmonary disease from virus administered intraperitoneally It might be expected that a virus which produces its effect so rapidly would penetrate the cell rapidly, as other viruses appear to do Further evidence that it may not is seen in the observations of numerous workers that several hours after the intranasal administration of virus, immune serum given by the same route may prevent significant infection in mice Moreover, Magill and I were able to show that influenza virus which had been exposed to susceptible cells in tissue culture for an hour, at least, could still be neutralized by immune serum On the presumption that antibody does not penetrate the cell these various facts point to a continued existence at the surface It may be that the surface stage is the period of essential damage and that cytolysis is a secondary autolytic phenomenon unrelated to intracellular virus and that the virus may obtain at the surface the materials required for multiplication

To return from this digression, how does poliomyelitis fit into the group pathogenetically? The idea that the initial infection of poliomyelitis virus is of the alimentary tract has been accumulating support The presence of virus in the mucosa of the pharynx and intestines strongly suggests a superficial localization although the higher incidence of paralytic disease in younger age groups may be an indication that first experiences are frequently more penetrating

Accepting the idea that antibodies in the blood represent previous experience with a virus of poliomyelitis, the fact that more than one infection can take place is seen in the observations that antibodies may be present in the blood at the time clinical disease is recognized Virus in considerable amounts may be demonstrable in the stools of apparently well individuals with circulating antibodies, indicating that propagation of the virus is going on in tissues unaffected by antibody of the blood Moreover, in monkeys second infections can be frequently instituted under a variety of conditions In general, while the data indicate that recovery or growth does reduce the probability of severe disease, repeated infections of the superficial alimentary tissues may be experienced

Rabies is not a superficial infection and does not belong in this group Nevertheless, the virus is ordinarily introduced by bite directly into the nervous tissue where it may escape circulating antibody On the other hand, proper vaccination with inactive virus appears to have a protective effect, although it is not clear how much of the effect of active virus may be related to an interference rather than to specific immunity Immune serum given into the area of injection of virus has been shown to be effective in protection, again by countering virus at the initial site

There is one additional factor in this group of virus infections which adds to the difficulty of maintaining permanent immunity That is the *existence of* antigenically variant strains The significance of certain minor variations is not at all times clear but the broader type differences are unquestionably clear

And so the superficial type of localization which appears to limit the efficacy of antibody unless available at that site and the existence of multiple strains of virus combine to give temporary immunities

Group 4 Virus Diseases in which both Virus Infection and Immunity Persist Independently In those instances of tumors definitely incited by viruses the circumstances differ from any of the other groups discussed—although resembling some features of Group 1—in the continued stimulation which they apply to cell growth It is commonly accepted and the serological data support the idea, that virus may be transferred through numerous generations of cell division while antibodies in the surrounding fluid are helpless except when cell destruction takes place, a state which Rous has termed a clandestine relationship Nevertheless, the antibodies are capable of limiting extension of the process as is shown by the fact that with Shope papilloma the tumors do not readily migrate to the adjacent areas of the skin Moreover, there is seen at times a sudden regression of growth indicating that immunity has gained ascendancy Green has recently suggested that in the tumors the virus actually hybridizes the cell so as to alter its specificity and make the virus antigen the dominant characteristic In the mouse mammary cancer, immune serum added beforehand inactivates the presumed virus which resides within the cell transplants Perhaps to the present discussion the most pertinent point is, however, that among the virus tumors persistence of virus is associated with active evidence of infection circumscribed by a demonstrable, persistent immunity and that the limited effectiveness of antibody is related to the mechanism of virus action The manner in which the two agencies, virus and antibody, exhibit their respective influences almost independent of one another further indicates that complete immunity is not derived from active infection and persistence of virus

COMMENT

In general, it appears that immunity in virus diseases is, as with infections by other agents, related to the interplay of antibodies and the cells of the body, varying according to the mechanisms of infection and the characteristics of the agent In the instances when virus becomes entrenched in, and protected by cells, antibodies can serve only to prevent the free dissemination of virus by meeting it when it emerges from the cells and by gradually reducing the focus to the point of obliteration In those circumstances, antibodies to be effective must be maintained at a high level and, if a disturbance in the host's functions is initiated by physiologic insults, the agent may gain the ascendancy through a reduction either in antibody production or in the capacity of the disposal mechanisms to remove the sensitized agent, or both In most of these instances the important item is not merely whether antibody is present in the blood alone but its concentration and its availability to the tissues where the virus is encountered This fact is shown by the relative efficiency of immunity according to the route employed for its testing in experimental animals When unphysiological routes, such as direct inoculation into susceptible brain cells, are employed the degree of immunity is low since virus may be enabled to avoid interstitial antibody

When routes are employed which give acquired antibody an opportunity to come into action its effectiveness is enhanced and greater protection results Morgan, Schlesinger and Olitzky, in a series of studies, have shown the variation in the quantitative relationships between the amount of antibody required for protection and the method of testing against the virus of Western equine encephalomyelitis In order to get detectable antibody in the spinal fluid of rabbits antibody in the blood had to be pushed to high levels, and in these circumstances protection against intracerebral inoculation was achieved This is similar to the probable behavior of antibodies in furnishing protection to the respiratory epithelium in influenza

The efficacy of vaccination with inactive viruses is also more reasonably estimated when challenge is made by normal physiologic approaches In this respect, from consideration of the mechanisms of infection one might predict that diseases in which a blood stream phase is essential to full development are those in which immunity could be most readily induced The results of passive immunization with measles are a clear invitation to active immunization The fact that gamma globulin is effective in prophylaxis of infectious hepatitis indicates that the blood stage is an essential part of its pathogenesis and that vaccination should be effective The encephalitides, already mentioned, dengue and others of that group are similarly inviting In other conditions the effect will depend upon the amount of antibody that can be made available locally at the portal of entry Protection of the local area where the virus makes its entry may protect the body as a whole

It is not intended to imply that continued infection may not influence antibody levels or that cells may not be altered in their receptivity to virus or in their capacity to dispose of virus Cells may be rendered refractory to infection by virtue of their being infected, as with interference, or by alterations in physiological reactivity which create a non-specific impermeability as is seen to some extent after physical, chemical or developmental stimuli There seems to be, however, no more need to call upon the virus to persist in order to have continued production of antibodies than in the case of soluble toxins or egg albumin After the type is set the printing press continues to print

To summarize, then, antibodies are effective in virus immunity according to the invasive mechanism involved, the type of parasitism and the availability of antibody at the portal at which virus makes its entrance to the body The behavior of different viruses and their diseases can be interpreted according to these factors, and the behavior of immunity in others can be predicted when certain of the mechanisms are disclosed The need for physiologic conditions of testing for proper evaluation of immune mechanisms is becoming increasingly apparent, emphasizing that it is not sufficient that antibody be present but that there be enough in the right place at the right time

PROBLEMS OF AUTOTROPHY[1]

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"Nature in her unscrutable wisdom has
set limits which she never oversteps—"
Jean Ray (1630)

It is indeed a privilege to discuss some aspects of the problem of autotrophy At first sight this topic may appear to be somewhat remote from the urgent problems of bacteriology, a subject suitable for those fortunate few who are free from the demands of industry, agriculture, or medicine But, in reality, this is not the case and we shall soon be squarely in the midst of basic questions of bacteriology, and it is to these fundamental principles of the science that the study of autotrophy can contribute

The term autotroph means "self-sufficient" but is has been applied in a more restricted sense to a very small group of bacteria (3, 5, 28, 33, 36, 46, 47) Since there has been some confusion associated with the name it is well to delineate precisely what is meant Strictly autotrophic bacteria are those bacteria distinguished from all others by two properties

1 Their sole source of energy is the oxidation of an inorganic material This material is irreplaceable In reality there are only four clearly established types of strictly autotrophic bacteria The *Nitrosomonas* species and related forms which can oxidize ammonium salts, the *Nitrobacter* species and related forms which can oxidize nitrites, *Thiobacillus thiooxidans* which oxidizes sulfur under acid conditions, and *Thiobacillus thioparus* and related forms which oxidize sulfur under alkaline conditions There may well be many other strictly autotrophic bacteria but those listed have been established without doubt In all of these cases the material oxidized is specific and cannot be replaced by any other substance The strictly autotrophic bacteria are not the most adaptable type of cell, as has sometimes been supposed (46) It is true that they live in somewhat odd environments, but any ability to adapt themselves to any other environment has yet to be demonstrated All of the well-established strictly autotrophic bacteria are limited for their energy source to the oxidation of a single specific inorganic material

2 Carbon dioxide is the sole and irreplaceable carbon source The important point is not that the autotrophs are capable of using carbon dioxide, for practically all living cells can do this, (37, 47) nor even that carbon dioxide can serve as the *sole* source of carbon But the basically important thing is that nothing except carbon dioxide can so be utilized It is a specific requirement, no other carbon compound yet known will replace carbon dioxide as a source of carbon for growth

[1] Address to the Society of American Bacteriologists by the recipient of the Eli Lilly and Company Award in Bacteriology and Immunology Philadelphia, May 15, 1947

The life of the autotroph is therefore a limited one but it is in these very limitations that its study may be valuable These are bacteria in which each has a single specific energy source, and from the energy so derived they not only can but *must* synthesize their entire cell substance from carbon dioxide

But even this is not all, for among the strictly autotrophic bacteria there is one whose properties are such that if we did not know them to be actually the case, we would consider such properties impossible This organism is *Thiobacillus thiooxidans* It was discovered by Waksman and Joffe (43) some twenty-five years ago and the studies of Waksman, Starkey, and their associates (31, 32, 44, 45) have firmly established the following knowledge of its properties *Thiobacillus thiooxidans* oxidizes sulfur to sulfuric acid at an acid pH range extending *downward* from pH 5 to a pH of 0 or less It will not only survive (a property common to many bacteria) but will actually grow in 5 to 7 per cent sulfuric acid. During sulfur oxidation in a purely inorganic medium, carbon dioxide is fixed to form the substance of the cell

If carbon dioxide is excluded, but sulfur is supplied, no growth occurs Nor can any material yet studied replace carbon dioxide as a source of carbon If sulfur be excluded but carbon dioxide supplied, only thiosulfate and perhaps hydrogen sulfide can serve as an energy source, and both of these, under the acid conditions necessary, are rapidly and spontaneously converted into sulfur No material other than sulfur suffices as an energy source, no material other than carbon dioxide is suitable as a carbon source

Here, then, is an apparently unique form of life since there is no other organism known to science which

can live in a purely inorganic environment,
can oxidize a specific inorganic material for energy,
can manufacture its entire cell substance from carbon dioxide and nothing else, save mineral salts,
cannot utilize any other materials for either energy or carbon,
and which not only can, but must, grow under acid conditions which are decidedly toxic to most other forms of life

If we did not know that such an organism existed, we would consider that life under these conditions was quite impossible, for more drastic conditions for the conduct of life can hardly be conceived Basically we consider it impossible because we have, in the vast body of knowledge accumulated in bacteriology, certain basic principles, certain conditions which must be met before life is possible, certain ways in which an organism, of whatever type, carries out its life functions We realize, consciously or unconsciously, that nature has set certain limits which she never oversteps, yet, *Thiobacillus thiooxidans* apparently violates these principles—it acts outside of nature's limits Is it possibly a different type of life? Is it a surviving example of the first forms of life to develop, as supposed by some? Or does it too conform to those basic principles which hold for all other forms of life?

A study of these principles has been termed by Kluyver "comparative biochemistry" It is the function of this embryonic science to *investigate* the

problem of whether or not there exists a fundamental unity in all living forms and, if such a unity is found, to define its nature. Because there are different degrees of similarity and varied kinds of differences among organisms, knowledge of the existence of such a unity has always been somewhat intuitive. To our minds, what has been principally lacking is the study of a test case. If one could find an organism which was so obviously different from other forms of life as to be a source of wonder that it could live at all, one could then apply to this organism the deductions derived from more normal forms and thus test their universal significance. One could, perhaps, define the limits which nature has set and which she never oversteps. We therefore began to study *Thiobacillus thiooxidans* with the hope that it would lead us to a definition of these limits in a *deliberate* attempt to test the general principles of bacterial metabolism.

I propose to examine briefly only a few such principles, omitting, for purposes of brevity, extensive documentation or description of experiments. For example, *Thiobacillus thiooxidans* apparently violates the general principle that the living cell (of whatever kind) can obtain energy *only* from reactions carried out at or within its boundaries. Most food materials are soluble, they enter the cell and are there acted upon under controlled conditions. The insoluble materials are normally acted upon by extracellular hydrolytic enzymes or are phosphorylated at the cell surface. The reactions which yield energy to the cell occur within the cell or at its boundaries. But sulfur is so insoluble in the media in which the thiobacillus can grow that its solubility has never been detected. Nor can it be hydrolyzed to simpler materials. Here, then, is a critical test of the universal nature of this principle.

In the case of the thiobacillus, Vogler (41) demonstrated that in order to oxidize sulfur, a direct contact between the organism and the sulfur particle was necessary. If separated by a cellophane sac, for example, no sulfur oxidation was possible. The sulfur oxidation was proportional to the surface of the sulfur available and microscopically, as had been recorded many times before, the bacteria could be seen clinging to the sulfur particle. We (34, 35) later offered an explanation of the ability of the thiobacillus to oxidize sulfur by demonstrating that the bacterium contained a fat globule composed of a highly unsaturated fat (in which sulfur would be soluble) which it placed in contact with the sulfur thus dissolving it and bringing it into the cell for controlled oxidation. *Thiobacillus thiooxidans* did not therefore violate the general principle that reactions yielding energy to the cell must occur at or within the cell. It apparently carried its own solvent with it. The cytological studies of Knaysi (11) confirmed these observations in part but added the observation that the organism was encapsulated which would render the fat solubility theory dubious. Unfortunately, our own observations do not agree with Knaysi's and careful reinvestigation has failed, in our hands, to demonstrate capsulation. The basic action of mechanism is thus still in doubt but the observation of the necessity of the direct contact between the organism and the sulfur particle, which is agreed to by all, demonstrates that these organisms do not violate the principle of oxidation at or within the cell.

A basic conception of the use of oxygen by microörganisms and higher cells as well, is that oxygen enters into the reaction only at the end of a relatively long chain of oxido-reductions, the most important of these, and perhaps the only one yielding energy directly to the cell involves the cytochrome or Warburg-Keilin system. The energy is derived from sulfur in exactly this manner (40). The energy of activation is 19,900 cal. determined experimentally, the pressure of oxygen has no effect, the system is extremely sensitive to azide, cyanide, and carbon monoxide (the latter being light reversible), in short, sulfur is oxidized by a typical cytochrome system.

This type of observation actually emphasizes two other basic principles and, with other data to be cited clears up, as far as it can be done, a misconception which has long been associated with the autotrophic bacteria. The first principle, which initially may seem surprising, is that the oxygen taken up in the oxidation of materials by the cell appears not in the oxidized product but originates from the water. The air we breathe in is converted to water, the oxygen in the carbon dioxide we breathe out comes from water. In this case the sulfur is oxidized by an electron-carrying system, the cytochrome system. The last step in this reaction is to combine hydrogen ions, electrons, and oxygen to form water. The sulfur unites with the water to form sulfate. Here is an illustration of this principle uncomplicated by the usual intermediate oxido-reductions present in the metabolism of organic compounds which have so long obscured this basic fact.

It is, of course, quite impossible to determine as yet whether the electrons are removed from the sulfur before or after its combination with the elements of water. It is thus still possible that life exists on a "flame of hydrogen," or rather on a stream of electrons originating from hydrogen. In sulfur oxidation, if hydrogen is removed after combination with water, the origin of that hydrogen must have been the water itself.

The second principle is that the passage of electrons through the cytochrome system can generate energy-rich phosphate bonds. This has been, in heterotrophic tissues, extremely difficult to demonstrate since the studies have been complicated by too many other reactions. In the thiobacillus, however, the reaction appears to be clear enough. Sulfur is oxidized by the cytochrome system and although the reaction is undoubtedly complex, it appears to be a mere passage of electrons from the sulfur to oxygen. The result of this oxidation is the formation of energy-rich phosphate (42) so that the passage of electrons through the cytochrome system *can* generate energy-rich phosphate. There is thus the very good possibility that the extra energy-rich phosphate found in the oxidation of organic metabolites in heterotrophic tissues owes its origin to the ability of the cytochrome system to generate energy-rich phosphate.

The misconception referred to, fortunately not widespread, is that because of the ability of the autotrophs to live in a purely inorganic environment they must be the surviving examples of the first form of life, that they must be primitive forms (3, 5, 28, 33, 36, 46, 47). Back of this misconception is the fallacy that simplicity of form and simplicity of nutrient requirement means

simplicity of metabolism The presence of a relatively complete and functional cytochrome system is indicative of a complex and highly developed metabolic process and, together with further data to be cited, effectively disposes of any notions of the primitive nature of autotrophic cells

There was another rather odd misconception of autotrophic metabolism current at the time we began these studies It had been supposed by some that because the growth requirements were exceedingly simple, the metabolism of this form of life was not comparable to that of heterotrophic tissues (4, 5, 33), that the chemosynthetic mechanism (which is the synthesis of all cell substance from carbon dioxide in the dark) was a unique process furnishing the cell with all necessary materials in a manner different from that of heterotrophic tissues Indeed, the cell was supposed, by some, to have no organic metabolism of its own since it was quite incapable of utilizing organic materials supplied to it (21, 22, 23)

Upon investigation, however, the cells were shown to possess a metabolism in the absence of oxidizable sulfur (38) Boemke (4), quite independently, demonstrated the same fact for nitrobacter In the thiobacillus this metabolism consisted of a breakdown of a previously synthesized polysaccharide The actual course of the metabolism (12) was quite similar to that of the heterotrophic tissues O'Kane (26) demonstrated the presence of several of the B vitamins in the tissues, and it was later shown that the nicotinic acid was present in the form of coenzyme I (15) LePage (12, 15) demonstrated that the polysaccharide breakdown involved in essence the same phosphorylated intermediates as were found in heterotrophic tissues These studies depended for their success upon the development of methods for the estimation of the phosphorylated intermediates in relatively micro amounts These methods were first developed by O'Kane (25, 27) and brought to a very precise and adequate basis by LePage (17) who has later made extensive use of them in the study of shock in animals (13) By their use LePage (14) was also able to isolate phosphoglyceric acid from the clostridia in which the presence of phosphorylation had remained in some doubt because of the earlier failure to obtain this intermediate

From the data cited it seems obvious by now that the autotrophic cell obeys the same laws as do other living cells And with it we have come some of the way toward defining those limits which nature has set and which she never oversteps Yet back of the problem of autotrophy is the still greater problem of synthesis, and there has emerged from these studies a clearer view of one of the basic principles of biosynthesis The late Dr Vogler, who was killed in the fighting for Java, formulated it in the following words as early as 1941 "No energy can be transferred from one of two coupled reactions to the other unless a common compound links those reactions together" And, experimentally, there is its corollary that the common compound which links coupled reactions is a compound of phorphorus Another way to state this concept is that there is interposed between the energy yielding reactions of the cell and those utilizing energy a compound common to both and this substance is a compound of phosphorus

At this juncture it appears to me reasonable to reiterate what has become evi-

dent to many workers (10, 18, 19) who have considered closely the production and utilization of energy by biological rather than chemical systems, namely *that frequently the free energy released by a chemical reaction occurring in a cell is not available to that cell.* The free energy released by a chemical reaction is a measure of the ability to do work but whether that work is actually done depends upon the presence of a suitable mechanism for utilizing the free energy. But the living cell is somewhat limited in the mechanisms it can employ to utilize free energy. It is not a heat engine, it does not utilize a mechanical contrivance, it is not an electric motor, it does not, in fact, conform to any of the physico-chemical devices which man has been able to construct. One might well make use of the concept of "biological free energy" as being that type of free energy which is available to the cell.

This is, in essence, the meaning of the concept of a common compound linking reactions in biological systems. The living cell can make use of energy released within its boundaries only if it has a suitable mechanism for trapping this energy. In so far as we know anything about these matters, the device which the living cell uses to trap the free energy released is to have the same compound taking part in the reactions releasing energy as takes part in the reactions utilizing this energy. In short, if a reaction A + B going to C liberates energy and a reaction D + E going to F requires energy, the only way that we now know by which a living cell can use the energy from A + B to run the reaction D + E is to have compound X in both of them. X absorbs the energy from the A + B reaction and holds it in its own molecule. This material may move some distance from its site of origin to give up its energy to reaction D + E. This is not to say that such is the only device which the cell employs *but that it is the only method which we now know,* whereby the energy can be transferred in biological systems. Certain reactions yielding energy are incapable of reaction with X, and in these the energy released is lost to the cell. It is not "biological free energy."

To return to the autotroph, in the study of sulfur oxidation Vogler (39) discovered a very curious thing. If the thiobacillus was allowed to oxidize sulfur in the absence of carbon dioxide and was then placed under conditions (in nitrogen, for example) in which such sulfur oxidation was impossible, it would fix carbon dioxide. In short, if sulfur was oxidized without carbon dioxide, the energy resulting from this oxidation could be held in the cell for a while and later could be used to fix carbon dioxide. Vogler (42) was then able to show that during oxidation of sulfur in the absence of carbon dioxide, phosphate was taken up by the cell and during the fixation of carbon dioxide in the absence of sulfur oxidation, phosphate was released by the cell. On sulfur oxidation the energy was converted into energy of phosphorylation, on carbon dioxide fixation the phosphorylated energy was released. There was a common compound linking the energy producing reactions (sulfur oxidation) with the energy utilizing reactions (carbon dioxide fixation) and this compound was a compound of phosphorus. The concept of a coupling of oxidative and assimilatory reactions could now be broadened to include the process of carbon dioxide fixation.

LePage and I (15, 16) were later able to isolate and identify the phosphory-

lated compound common to these reactions. It proved to be an adenosine triphosphate hitherto unknown, similar in some of its properties to the adenosine-5-triphosphate, the ATP of animal tissues and most bacteria, but strikingly different in other properties so that, by comparison with the properties of a ribose-3-phosphate, we concluded that its structure was that of adenosine-3-triphosphate (16). We did not, however, consider the possibility that other structures, for example, arabinose-5-phosphate, might have similar properties. The important point at the moment is that the adenosine triphosphate isolated was a new one, differing from that common to heterotrophic forms. This point, of interest at the time, had no particular meaning until recent studies, which I shall soon cite, brought it again into prominence.

After this, it was almost inevitable that we should consider the greatest synthetic reaction of them all, photosynthesis. Perhaps one of the troubles with studies or theories of photosynthesis was that the process had been regarded as a unique one whereas it was merely a modification of something that nature had used before and was familiar with. If we consider the animal or most bacteria, the energy is derived from the oxidation or fermentation of organic materials, the energy is utilized for the synthesis of the cell, and the link between them, in so far as we know these matters, is energy-rich phosphate (10, 18, 19). In the thiobacillus the energy is derived from the sulfur oxidation, is used for the synthesis of the cell from carbon dioxide, and the link between them is again energy-rich phosphate (15, 16, 42). In the photosynthetic organisms the energy is derived from the absorption of light and is used for the synthesis of the cell from carbon dioxide. We therefore considered whether it was possible that the link between *these* coupled reactions was energy-rich phosphate, and, finding nothing which would eliminate this possibility, proposed (6), as a working hypothesis, that, in common with the rest of nature, photosynthesis employs the energy-rich phosphate bond as its energy carrier. We conceive that the result of the absorption of light by the chlorophyll system is the formation of energy-rich phosphate, that the other reactions of photosynthesis (oxygen production (7), carbon dioxide fixation and reduction and cell synthesis) could be accomplished without use of light if one could substitute, into the system, the energy-rich phosphate compound which results from the action of light on chlorophyll. Now this hypothesis is a little bit radical, to put it mildly, and will undoubtedly have an even more hectic career than it has already experienced. It has been roundly condemned (9, 29), tolerated (8, 20), and even sometimes considered as within the bounds of reason (19, 24, 30), but to our minds its chief virtue lies in the suggestive method of approach to the problem of photosynthesis or, in fact, to other syntheses in which energy is involved. Admittedly, but little progress has yet been made in this direction, but the intervening war years have not been favorable for such studies. A sign that progress can be made in this direction, however, comes from the recent studies of Dr. Albaum (1, 2) who has demonstrated that plant tissues and perhaps photosynthetic bacteria contain an adenosine triphosphate differing from the animal type and closely resembling that from the thiobacillus. It thus appears that those organisms, either chemo-

synthetic or photosynthetic, whose sole carbon source can be carbon dioxide, possess an adenosine triphosphate differing from the animal or other bacterial tissues Whether this is a basic and fundamental difference, or purely circumstantial, remains for future research

The study of autotrophy is not, then, as remote from the problems of bacteriology as we might possibly suppose and its study can contribute to our basic knowledge For it is the knowledge of nature as she is, and not as we imagine her to be, that constitutes science In the very desperate situation in which the autotroph is forced to carry out its life, the basic principles which govern all living forms stand out with greater clarity

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SOME INTROSPECTIONS ON MOLD METABOLISM[1]

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Some two hundred years ago, it is said (7), Albrecht von Haller, famous Swiss scientist, described fungi as "a mutable and treacherous tribe" However limited the stimuli which induced von Haller to make this observation in those beginning days of the study of these organisms, modern students of the subject, with a vast background at their disposal, can only marvel at and attest to his astuteness Since von Haller was mainly a plant taxonomist and animal physiologist, and since his time was well before the inception of biochemical studies on molds (indeed, biochemistry in general), first firmly initiated by Raulin and Pasteur more than a century later, there is little doubt that it was the gross cultural aspects of fungi he was concerned with when he made his grim conclusion He could have had no idea of the tremendously wide application his verdict was to have on the then as yet unopened fields of physiological and biochemical and, in general, metabolic aspects of fungi And yet the same kind of frustration implicit in von Haller's succinct verdict is only too well known to the investigator of metabolism,—and with variations far beyond the scope of that versatile scientist's penetrating imagination

Time and mycologists have amply confirmed the incessant tendency of filamentous fungi toward spontaneous change, and this mutability has become recognized as one of the outstanding traits of this group of organisms Doubtless such mutability holds also for all microörganisms Its recognition first in the fungi probably has two explanations the gross size of these organisms in culture makes changes easily discernible, and structural differentiation of the organism into diverse morphological parts increases greatly the chances for visible deviation from parent cultures or from the norm

It was probably the industrial or applied microbiologist who was largely responsible for the emergence of the idea that in fungi there exists a mutability of even greater range and diversification than that observable by superficial inspection Detailed studies through the years on organisms of potential practical interest from the standpoint of their metabolic ("fermentation") activities have led to the following what may be considered as axioms in microbial metabolism, and especially in relation to molds

1 The individual progeny from any culture of single spore origin may vary within wide limits in regard to the performance of any given biochemical activity, despite the fact that all may be morphologically indistinguishable This phenomenon, known as strain specificity,[2] is not, with relatively few exceptions,

[1] The material in this article represents substantially the contents of a chapter from a book on mold metabolism under preparation by the author

[2] Incidentally, it may be pointed out that this conclusion, like so many "new" concepts and ideas in microbiology, and other sciences as well, turns out to be merely a rediscovery of a feature first recognized by a past master In this case it was the genius of Pasteur who

evident from simple inspection, but is revealed only by chemical analysis The degree to which this kind of mutability is so fundamental in theoretical and practical microbial metabolism is emphasized by the consideration that in addition to the range occurring with any one function, it must also occur individually with respect to every one of the host of biochemical reactions the organism is capable of effecting Thus the cells in any one culture are far from being homogeneous physiologically

When progeny with weaker power to effect a given biochemical function, get the upper hand in an initially potent culture, as they may in the course of continued successive transfer, the ultimately weakened culture is said to have undergone "physiological degeneration" Viewed in this way, the physiological potentialities of any culture are always changing during periods of active growth

With such differences possible within the progeny of any one culture, it scarcely needs to be emphasized how great differences may be expected between morphologically indistinguishable strains of diverse origins, such as isolates from natural sources, or from different stock culture collections Apart from *quantitative differences*, these often are even qualitatively different

2 In addition to the above, there is another kind of variation in progeny, and this is latent Two morphologically indistinguishable strains compared metabolically under any one set of conditions may, within experimental limits, respond so nearly alike that they might be considered physiologically indistinguishable Yet, tested together under another set of conditions, gross differences in metabolic behavior may become evident There are at this time few examples of this type to call upon among the fungi, but the data of Schulz (13) make a neat instance This author studied extensively the proximate chemical composition of the cell material of *Aspergillus niger* and found that the compositions of two different strains were virtually indistinguishable when cultivated on a certain basal medium The addition of a few ppm of zinc ion to this medium caused marked changes in composition of the mycelium, but the changes were strikingly different in the two organisms, so that there was no question that two different individuals were involved.

It is interesting to note that this procedure of discerning metabolic differences between morphologically indistinguishable organisms, actually forms, to a great extent, the basis of diagnostic bacteriology The principle is the same,

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in 1876 fully perceived, appreciated and even defined strain specificity, only he called it "le polymorphisme physiologique" (12a) In previous works the present writer has, unknowingly, been equally guilty with many others of this particular "rediscovery", full credit for which, we hope, will henceforth be ascribed exclusively to whom it belongs—that French savant

"On pourrait croire que toutes les variétés de *mucor* sont propres à donner le genre de levûre dont nous venons de parler Il n'en est rien C'est encore une preuve frappante des différences physiologiques profondes que peuvent offrir des formes de végétation pourtant si voisines que les classifications botaniques sont contraintes de les rapprocher autant qu'il est possible Déjà les *mycoderma vini* et les levûres alcooliques proprement dites, si semblables de formes et de développements qu'on les jugerait identiques, au moins dans l'état de nos connaissances, et si différents physiologiquement, donnent de ce fait un exemple extraordinaire" (12b)

—two organisms behave alike in their response to different physiological tests, and differentiation becomes possible only after a sufficient number of tests are applied so that eventually the sought-for difference in response materializes In bacteriology, such latent properties, ultimately revealed, provide the basis of species differentiation, and sometimes even genus separation Fortunately, the systematics of fungi is firmly based on morphology, for if latent physiological characters were employed as a means of species differentiation, the numbers of species would truly be infinite

The reasons why such diagnostic techniques are workable with the bacteria and not with fungi, are manifest to the microbiologist Diagnosis or differentiation is based largely upon the plus or minus ability of bacteria to attack certain individual (saccharidic) compounds as energy sources Similar exhaustive differential testing has not been done with many strains of any given species of fungus because such a feature has little diagnostic value in a system based exclusively on morphological criteria for identification The important feature of mold strains is the intensity with which individual strains carry out particular biochemical transformations As brought out in the next paragraph, fungi are extremely sensitive and unstable in this respect, whereas the bacteria as a class are much more immune to environmental circumstances and can generally be counted on to effect a stable and reproducible dissimilatory metabolism

3 The first two axioms of variation are based on differences inherent between different fungus individuals, and there is good reason for believing that they are of genetic or nuclear origin This is, then, genetic variation The third axiom is predicated on a different kind of variation, one quite apart from genetic differences between individuals This relates to the extreme susceptibility of the physiological potentialities of any given fungus culture—which, because of the enormous number of cells, may be considered collectively to function as an individual—to relatively slight alterations in environmental factors We may designate this as "response variation", and the discussion throughout this article deals with responses in a metabolic sense The term "physiological variation" is often employed, but it does not have the connotation that the former term does in excluding genetic phenomena and in limiting the meaning to environmental sensitivity

The unique and tremendous response differences, both quantitative and qualitative, obtainable with any single fungus culture as a result of imposed cultural conditions are so common an experience and so well known to the microbiologist that it is not worthwhile going into specific detail here, as it is more the fundamental cause we are concerned with than the effects However, for an orientation of the reader there may be cited biochemical characters such as amount and composition of cell matter synthesized, formation of extracellular enzymes, production of low molecular weight synthetic compounds (e g , antibiotics, vitamins, pigments, etc), accumulation of sugar split products (carboxylic acids), carbon dioxide evolution, and so on Generally all of these are in balanced interrelation, a change in one usually being reflected by changes in others

In passing, it may be mentioned that the alterations in cultural conditions which can induce most significant metabolic changes are often so slight that they are exceedingly difficult if not impossible to control fully, despite rigorous precautions This accounts for the difficulties in reproducibility of results between different laboratories employing identical cultures, and even between different trials in the same laboratory (penicillin yields still fluctuate within disturbingly wide extremes)

The remainder of this discussion, and the next three sections especially, is an attempt to analyze some of the intimate aspects of mold carbohydrate metabolism with the object of synthesizing a coördinating and interpretative scene from episodes which, it seems to the writer, have a bearing on Axiom *3* above If they are critically evaluated in this manner, the author will have achieved his objective, namely, the stimulation of thinking towards generalized concepts in the field of mold metabolism which hitherto has been deplorably neglected in this respect Admittedly, certain of these ideas may be considered by some to be premature, distorted, and incomplete If my colleagues and others consider this essay too speculative for their scientific natures, my defense rests on two licenses namely, the title of this essay, and the quotation of A. V Hill which Kluyver used in defense of his (Kluyver's) speculations and attempt at synthesis of the field of bacterial metabolism sixteen years ago "It is dangerous to speculate too far, but it is foolish not to speculate at all "

EFFICIENCY OF CELL SYNTHESIS OF MOLDS IN RELATION TO NATURAL ENVIRONMENT

It has already been stated that the metabolism of any given mold culture is dependent upon environmental conditions, and that it can be made to fluctuate between extremely wide limits so that the change may actually take on the appearance of a qualitative difference Indeed, it is a common event to have an organism produce no detectable amount of a particular metabolic product, and yet under different cultural conditions, produce that very substance abundantly Finally, there is the situation in which, on the one hand, one kind of product is produced, and, on the other, a totally different product What is the explanation of such behavior? While no single interpretation may provide the full story, it seems as though sufficient experimental and circumstantial evidence is available to provide a start on this intriguing question

In addition to the above mentioned major metabolic differences between filamentous fungi (molds) and bacteria, it may be pointed out that with possibly occasional isolated exceptions there are no anaerobic molds It is doubtful if obligately anaerobic molds exist Indeed, there is general concurrence with the idea that molds are highly oxidative organisms This is not say that molds with not metabolize carbohydrates anaerobically (fermentation), but this activity is accomplished by preformed cell material.

Even though reduced products are formed in aerobic cultures, e g , ethanol, they are without a doubt formed by cells deficient in oxygen Under the usual conditions of cultivation, some always are It is questionable if molds can de-

velop, that is, *grow* at a significant rate in the complete absence of molecular oxygen Certainly such exceptions are exceedingly rare

The possession by many molds of strong glycolytic mechanisms is, as seen below, the outstanding metabolic characterization of many of these organisms On the other hand, possession of strongly aerobic metabolism has a profound implication for the economy of the mold organism insofar as utilization of its available energy source goes It means that the organism has innately the ability to utilize most efficiently its substrate for growth and cell synthesis due to the fact that aerobic respiratory processes are the most efficient for liberating and utilizing, for multifarious cellular activities, the energy of the substrate which we shall assume is carbohydrate Considering the conditions prevailing in nature, such as in the soil, the natural habitat for molds, the idea of organisms possessing a type of metabolism which enables them to utilize substrate efficiently and to build up as much cell material as possible seems not unlikely

Due to the rather frugal and precarious nutritional environment prevailing in the natural habitat of molds, it appears not unreasonable that they have become adapted to survival and existence under threshold nutritional conditions by their high efficiency of utilization of the limited energy source available

The nutritional level of the soil must at most times be very low, except during those relatively isolated periods when fresh plant or animal residues are available Even then in neutral soils the fast-growing bacterial, and to some extent actinomycetal, population accounts for the destruction of the bulk of the readily utilizable material The fungi come in, then, as secondary invaders, utilizing certain of the more resistant components of the organic matter and the remains of other microbial cells Thus, through the competition with the rest of the soil population for available foodstuffs and the slow utilization of resistant organic fractions in the soil, the molds may be thought of as having become adapted through natural selection (possibly preceded by mutation) to a highly effective economy in their prevailing environment, e g , a low level or marginal nutritional state with respect to available energy and carbon source Such an effectively economic metabolism might account for the survival of and the high number of fungi occurring in normal soils This superior character could, then, be interpretable as a consequence of the failure of the organisms to waste energy in the form of products of anaerobic or aerobic metabolism, giving the efficient molds survival advantage over inefficient ones, which eventually would become extinct

The important consequence of the efficiency concept is that maximum efficiency of energy utilization by the mold is attained only when the substrate-carbon is converted entirely into only two products, namely, the components of protoplasm and the inevitable CO_2 There is no evidence that molds form any dissimilation products under normal soil conditions, and in line with the foregoing, the only reasonable conclusion is that molds in soil oxidize these substrates entirely to CO_2 and H_2O, aside from that relatively high fraction converted into cell material This concept probably has general application to the majority of aerobic microörganisms under the nutritional conditions

prevailing in soils most of the time,—complete conversion of substrate to CO_2, H_2O and cell material Exceptions would be those relatively few cases where split products of certain complex naturally occurring compounds would accumulate Such substances could not be attacked by the organism under any circumstances, and are not to be considered as dissimilation products, inasmuch as usually they consist of unchanged portions of the substrate molecules, rather than products arising from the substrate through transformations brought about by intermediary metabolism, and, in most cases, they resemble structurally the original substrate molecule As an example, one might cite the oxidation of the side chain of an aromatic compound, leaving the ring structure intact. In any case some organism could be found to decompose the compound completely

The inefficiency of anaerobic organisms in the utilization of substrate is a consequence of their leaving always a portion of the substrate in the form of metabolic reduction products, or expressed differently, they leave the major portion of the energy of the substrate in the form of organic metabolism products Obviously, with less energy obtained, the growth efficiency is reduced The same line of reasoning holds true in the case of metabolic products produced by molds as a result of aerobic consumption of carbohydrate, namely, organic acids and other excretion products Energy left in the form of accumulated products of any kind, actually means reduced efficiency of energy utilization up to that state In most cases the products may be further attacked after the original substrate is depleted, and their energy utilized (see later)

Hypothetical anaerobic (true) fungi probably could never survive competitively with the anaerobic bacterial population of the soil in the utilization of fermentable carbohydrates

OVERFLOW AND SHUNT METABOLISM

Why then do molds produce from sugar large amounts of metabolic products other than cell material and CO_2, namely organic acids, carbohydrates, polyhydric compounds, etc ? The best explanation seems to be that the metabolism of the organism becomes deranged It becomes so to speak, pathological This pathological behavior is a direct result of the influence of abnormal environmental conditions [3]

Of greatest importance is the carbohydrate concentration Invariably laboratory media for the cultivation of fungi contain carbohydrates in concentrations far exceeding those that the organism ever would encounter in nature and to which the mold is adapted for maximum efficiency of utilization This luxury of excess sugar sets off a chain of events culminating in faulty metabolism of the sugar as indicated by only partial utilization of the sugar molecule, leaving

[3] This general idea had already been cogently expressed for activities of soil bacteria by the venerable Sergei Winogradsky in 1928 He emphasized that the activities of soil bacteria in the laboratory are no criterion of their behavior in the soil complex He called such laboratory cultures "domesticated hothouse organisms" Soil Sci 25, 37–43, 43, 327–40 (1937)

incompletely oxidized products accumulating in the medium, usually indicated as organic acids, although other products may also accumulate outside and inside the cells It would appear that the enzyme mechanisms normally involved in complete oxidation of the substrate become saturated, and the substrate molecules then are excreted and accumulate as such, or they are shunted to secondary or subsidiary enzyme systems which are able to effect only relatively minor changes in the substance, which then accumulates in its transformed state The latter mechanism is by far the most common The limiting or bottleneck enzyme systems are never those concerned with the initial stages of carbohydrate dissimilation, but are those which act on the substrate only after it has been brought through the stage of split products When the rate of attack on the original sugar molecule is limiting, obviously subsequent enzymes in the chain can accommodate in the normal way all the raw split product available to them, no diversion results, and no waste dissimilation products ensue However, when the rate of splitting the carbohydrate chain into smaller products proceeds faster than the subsequent enzymes can handle them, a metabolic shunt occurs, resulting in accumulation of waste products, or increase in other products produced only in small amounts normally The inability to metabolize rapidly intermediates, which then are diverted through abnormal channels, has many analogies in general biochemistry For example, in yeast fermentation, the enzymes normally giving rise to ethanol can by chemical treatment be made limiting or inoperative so that triose from sugar is diverted to glycerol instead of ethanol In animal as well as microbial metabolism, carbohydrate nutrition above that required for basal metabolism is diverted to fat, which represents deposits of condensed sugar split products which accumulate as fat when the normal oxidation enzymes are surfeited

In fact, the probability is good that metabolic shunts actually are the basis of the widespread practice of securing increased intensity of certain biochemical properties on the part of various organisms through mutation techniques, irradiation, etc Especially has this objective been sought in connection with the biosynthesis of industrially important compounds,—penicillin, streptomycin, itaconic acid, and others Spectacular success has been achieved with penicillin, and some moderate success with itaconic acid A logical interpretation for these yield increments is that genetically controlled enzyme systems active in normal cells, and which offer an outlet for some of the intermediary compounds of the cell, are eliminated in the mutants, making proportionally more of the intermediates available to the other intact enzyme mechanisms, one of which, on a random basis, happens to be of interest to the investigator It would be difficult to account for increased synthetic powers on any other basis

The best evidence in support of metabolic shunts is that, other factors remaining constant, the enzyme saturation can be demonstrated simply by increasing the concentration of carbohydrate In dilute sugar media, from 0 up to 0 5 to 2 0 per cent sugar depending on conditions, molds usually will yield no organic acids during the phase of active growth. This is an experiment approximating the nutrition of molds in their natural surroundings adequate minerals of all

kinds, sufficient utilizable N, and very low C/N ratio due to very low carbohydrate supply Soluble carbohydrate concentration in the soil probably never comes anywhere near 0 5 per cent Results from such experiments may be adduced as comparable to the happenings in the soil environment Next comes a sugar concentration range where very small amounts of metabolic products will accumulate, and this becomes larger in proportion to increased sugar concentration up to a maximum of 8 to 15 per cent for most fungi This parallel accumulation of acids or other products usually is interpreted simply as the effect of carbohydrate concentration Actually it is more a measure of sugar split products in excess of those required to saturate the enzyme systems involved in the synthesis of protoplasm and in the oxidation to CO_2 Essentially it is "overflow" metabolism

It is evident the metabolism of the mold in a culture may be quantitatively as well as qualitatively different as the sugar concentration falls as a result of consumption The final balance of products represents merely the resultant of all the changing processes The validity of Kluyver and Perquin's (10) observation that clearcut biochemical evaluation of a mold can be made only in a high sugar concentration and for such a short time that the sugar concentration does not change materially is an all too-little appreciated fundamental of mold metabolism

If now conditions are imposed which alter the content of or capacity of the bottleneck enzymes, it might be expected that corresponding alterations in the amount of split products diverted through shunt reactions would take place This is actually the case, and it is possible experimentally to vary the intensity of the shunt reactions within wide limits by controlling key enzyme systems The effect of sugar concentration has already been discussed, and other evidence supports the idea

An easy demonstration of these points involves the so-called resting cell technique, also referred to as the replacement method (Pilzdecke), incidentally, also first used by Pasteur For example, under certain conditions of cultivation where low sugar concentration is present in the medium, *Rhizopus nigricans* or almost any other mold, will grow rapidly and synthesize an abundance of cell material and form much CO_2 Careful tests on the culture filtrate fail to reveal the presence of even traces of organic acid If this "pregrown" mycelium is now placed in a solution containing the same concentration of sugar as originally present in the medium but, except for some $CaCO_3$ as a neutralizing agent, containing no other nutrient material, large quantities of an organic acid, in this case fumaric acid, are formed quickly and accumulate in amounts comprising a substantial portion of the sugar consumed That is, the identical cells which formed no acids from sugar during growth now form acids abundantly The situation here is a logical development of the theme given above During the growth stage, with an abundance of all nutrients essential for the building up of cell substance, the sugar split products are combined with nitrogen, sulfur, and minerals and built up into larger structural and functional components of cell material In the replacement experiment with sugar solution, the sugar split products cannot

be further converted into protoplasmic materials in conjunction with nitrogen, sulfur and minerals because the latter are absent Unable to be consumed through normal synthetic or growth channels, the split products are diverted and partially oxidized through supplementary enzyme systems, which happen to give rise to organic acids and, as seen later, possibly other materials Some CO_2 also is always formed, and doubtless some is converted to intracellular carbohydrate *via* oxidative assimilation

One recalls in this connection that the amount of organic acid formed per gram of carbohydrate consumed during the early stages of growth of molds always is less than that formed in a corresponding period during the later stages of incubation Only near the end of maximum growth, i e , when cell synthesis begins to slow down, are maximum conversion yields obtained, due to diversion of carbohydrate dissimilation through channels of acid formation

A further striking example in support of the metabolic shunt is provided by the elegant experiment of Beadle, Mitchell and Houlihan (1) in which the enzyme normally acting upon a metabolic intermediate is removed entirely by destroying the gene responsible for synthesis of that enzyme, i e , creation of a mutant differing from the normal parent only by lack of one specific enzyme A mutant strain of the mold *Neurospora crassa* was obtained which could not synthesize adenine due to lack of the enzyme essential for the conversion of adenine precursor to adenine Blocked in its normal outlet, the precursor is now disposed of in a manner apparently totally foreign to a normal strain,—it undergoes polymerization to form a purple pigment which accumulates in the mycelium and medium To all appearances the organism has acquired a character, yet in reality it is merely forced to use an otherwise latent enzyme system

Based on the foregoing, one of the best ways to test the latent ability of an organism to accumulate dissimilation products, is to provide it with excess sugar or other substrate and deprive it of one or more other nutrients essential to growth This applies, of course, only to preformed vegetative cell material Starting from spores, no growth would occur if an essential element were omitted One might accomplish similar results by providing only limited amounts of a certain supplementary nutrient so that it quickly becomes exhausted during early growth, then forcing sugar metabolism through the accessory metabolic channels

Another interesting demonstration of this idea centers around the catalytic effect which trace elements exert on the efficiency of utilization of available carbohydrate by molds Notable in this respect is zinc, and to a lesser extent iron, manganese, and copper The exact mechanisms by which these elements participate in mold metabolism are not known, but their overall effects have been established many times as catalyzing the conversion of substrate into cell material [4] If a few ppm of zinc ion are added to one flask of a zinc-deficient 2 per cent carbohydrate complete-mineral medium and another no-zinc flask of the same medium inoculated with any acid-forming mold, and the cultures analyzed after a suitable growth period of 5 to 10 days, some profound differences are apparent The zinc containing culture will have synthesized an abundance of

[4] For review see Foster, J W , 1939 Bot Rev , 5, 207–239

mycelium meanwhile producing no organic acid, or, at most, very small amounts The no-zinc culture gives the reverse picture, much smaller mycelium development and substantial accumulation of organic acid in the culture filtrate Also, in the zinc culture, an appreciably larger percentage of carbohydrate carbon ends up as CO_2 as compared to the no-zinc control

The explanation of these striking differences lies in the rôle of zinc, presumably functioning as a coenzyme, in catalyzing some reaction that permits more complete oxidation and conversion of the carbohydrate into cell material Remembering that this conversion necessitates utilization of sugar split products, it is evident that zinc functions in some way as a mediator of enzymes involved in the transformation of split products to protoplasm and that the presence of the right amount of zinc is the governing factor in these transformations In the zinc deficient culture the transformation enzymes can operate only inefficiently, hence the split products are diverted to organic acid forming enzymes

However, the fully efficient zinc enzymes, of sufficient catalytic power to handle completely the split products from a 2 per cent sugar medium, can themselves become saturated by the split products from a higher sugar concentration (5 to 10 per cent), so that overflow metabolism sets in, and considerable organic acid accumulation takes place in the presence of an amount of zinc-enzyme sufficient to repress acid formation from lower sugar concentrations

Further experimental confirmation of the metabolic shunt origin of products in mold cultures involves supplying artificially a dose of the precursors of a particular end product during the stage of enzymic saturation, i e , during active utilization of supra-minimal sugar concentrations Two Italian investigators (4) did this experiment, adding to cultures of *Aspergillus niger* equimolar quantities of malic and glycolic acids as citric acid precursors in accordance with one of the current schemes for the biological synthesis of citric acid, namely condensation of C_4 and C_2 acids In every case increased citric acid yields were obtained, as high as 9 28 times the amount formed from the sugar alone, when obviously the citric acid-forming enzymes were still unsaturated and were acting on split products or derivatives diverted from other saturated oxidative or synthetic mechanisms

Several other examples of shunt metabolism could be cited The well-known practice of employing poisons for specific enzymes is one This technique is such a common one that details will not be given except to emphasize that when a poison shifts the balance of products formed by a microörganism, it is in principle, effecting a shunt metabolism Normal enzymes are inhibited and subsidiary enzymes then come into play with greater intensity than otherwise One may cite also the accumulation of reduced metabolic products as an aerobic organism is deprived of oxygen In molds the reduced product generally is ethanol, while under good conditions of aeration, it is not formed, or, more often, in smaller quantities In the presence of oxygen, C_2 split products, *if formed* at all, are oxidized *in situ* to CO_2 and H_2O *via* flavoprotein and the cytochrome hydrogen transport system Deprived of this pathway, in absence of oxygen, C_2 split products function not as hydrogen donors for oxygen, but as hydrogen acceptors from triose, and become reduced to ethanol

It is understood that shunt reactions are in reality paired reactions which depend not only on a saturated and overloaded enzyme system, but also on a second enzyme system, normally latent or subdued, whose activity becomes manifest or accentuated through the availability of overflow intermediates Not unexpected, then, would it be to find instances where the latter enzyme system is lacking and, as a consequence, the overflow intermediate is not metabolized through a diversionary route One would look for the hypothetical intermediate to accumulate, inasmuch as there is no other way out Several examples of this type could be given A fine instance of this simplest kind of metabolic block occurs in a strain of *Fusarium lini* in which a natural cocarboxylase deficiency results in a retarded rate of pyruvate decarboxylation as compared to the rate of formation of this acid from carbohydrate, the pyruvate accumulating and being easily isolated from the medium Addition of thiamine to the culture medium restores the cocarboxylase level essential for maximum efficiency of carboxylase activity, eliminating thereby the enzyme bottleneck and pyruvate no longer piles up in the culture fluid (15)

From all the foregoing it is evident that the ability to form dissimilation products is intimately linked with the processes of cell synthesis and carbon dioxide production Resolved into mechanisms, the final balance depends on the capacity of the oxidative and cell synthesizing enzymes in relation to the load of carbohydrate split products they have to carry

MECHANISMS AND POSTULATED INTERMEDIATES

One of the most favored and time honored approaches to the problem of intermediary metabolism is to feed a biochemical system a series of chemicals postulated to occur somewhere between the breakdown of the substrate and the formation of the particular endproduct If the system utilizes the added compound and produces in reasonable yield the identical endproduct formed from the original substrate, the added substance is considered to be a normal precursor of the endproduct in the pathway from the original substrate Extensive use has been made of this technique in mold metabolism, particularly in relation to mechanisms of formation of organic acids Almost invariably the technique has been to employ the supposed intermediate as the only source of carbon in an otherwise complete medium, inoculate the organism and test for the particular endproduct in question after suitable incubation times Generally preferred is the technique of using washed, preformed mycelium furnished with the suspected compounds alone or with accessory nutrients

It is not the purpose of this article to judge the general validity or acceptability of this kind of evidence in biochemical work However in mold metabolism the situation is such as to warrant a few theoretical observations specifically applicable to this field Despite rather general use of this approach in the study of any one product, be it oxalic acid, kojic acid, citric acid or others, the results so far available are diverse and so conflicting that with few exceptions it is impossible to draw decisive conclusions as to the true mechanisms in question For example it is, on the surface, astonishing that such opposing data have been obtained per-

taining to the single process of oxalic acid formation in fungi, all with the "added intermediate" technique Thus some authors get abundant oxalate formation from acetate, others insignificant yields Some find and propose glycolic and glyoxylic acids as midway between acetate and oxalate especially on the strength of some conversion of these two acids to oxalate Others maintain oxalate results from a hydrolytic split of oxalacetic acid, the latter resulting from acetate condensation through the C_4 dicarboxylic acid system Careful experiments by a different worker fail to reveal any oxalate when oxalacetate is fed to the organism, instead this worker excludes acetate from any rôle in the process and postulates instead a hydrolytic fission of 2-keto gluconic acid to yield oxalate Others believe that acetate is split out of initially formed citric acid, and next are the experiments in which oxalate is formulated as originating by dehydrogenation of 2 mols of formic acid Finally no one has offered any mechanism for the extraordinary high yields of oxalate obtainable from peptone solutions One must remember, too, there is considerable arbitrariness as to whether a yield of the endproduct is of sufficient magnitude to warrant assertion that the tested substance actually is a precursor In some cases conversion yields of only a few per cent have sufficed to incriminate certain precursors, and yet other workers believe that the bulk of the precursor should eventuate as the product, else the reaction is a secondary side one

Controversial results like these typify other branches of mold biochemistry It is illogical, mainly on the basis of comparative biochemistry, to assume the existence of so many different mechanisms for the formation of a single organic acid resulting from carbohydrate breakdown There must be a flaw in the experimental approach, and a likely one stems from the concept of shunt metabolism

Worth reiterating here is the view expressed and implicit in the previous section that an organic acid (for example) is formed in quantity from carbohydrate only after the organism has satisfied its primary assimilatory requirements The precursors of organic acids are surplus over those requirements Now when a fungus is furnished a hypothetical precursor as the sole carbon source the likelihood is exceedingly strong that a significant portion, if not the bulk of the precursor, goes into the now unsaturated assimilatory or respiratory channel, or both, in which situation the precursor is no longer surplus And since precursors generally are compounds which would yield integral assimilation building blocks only inefficiently, a large amount of these compounds would undergo consumption and combustion to fulfill these primary needs of the organism, leaving little chance for direct conversion of precursor to product In such circumstances an actual precursor might be erroneously eliminated from consideration

It is entirely conceivable that differences in results obtained hitherto by various workers may be explained by the use of different strains of *A niger*, or other organisms, which on account of strain specificity, vary quantitatively if not qualitatively in the degree and efficiency to which their assimilatory and respiratory requirements are saturated Strain specificity doubtless explains the prevailing confusion

To put the experimental method on a basis consistent with theoretical concepts one must perform such experiments under conditions where the complicating assimilatory and respiratory processes are, so to speak, presaturated and hence minimize the importance of these phenomena in the independent conversion of precursor to product The most logical and efficient way of doing this is to have the organism actively metabolizing carbohydrate before and during the presence of the added precursor Consumption of precursor now should theoretically be largely via conversion to endproduct Obviously, optimum conditions would be those where the assimilatory reactions are saturated, and the system forming the particular endproduct unsaturated, so the latter can accomodate added precursor Hence the carbohydrate concentration should be sub-optimal for maximum product formation when tested alone, indeed, that concentration just beginning to manifest overflow metabolism by the appearance of small or moderate yields of product might be the best one to employ for the precursor additions

Adoption of such a technique, or at least the principles involved, might provide the means for obtaining more consistent results and in stabilizing what is presently a decidedly unsettled field Incidentally, it might be pointed out that on theoretical grounds at least one other possibility exists for eliminating the interference of assimilatory mechanisms in preformed mycelium, namely, through selective inhibitions by poisons A program testing these possibilities is under way in this laboratory

MAIN PATHWAYS OF CARBOHYDRATE METABOLISM IN MOLDS

Under this heading it is intended to *present a rationale* which in a general way will serve to coördinate what appear on the surface to be a host of complex and unrelated types of metabolism in the numerous molds so far studied in some detail If one invokes the precepts of comparative biochemistry first enunciated and brought to bear on microbiology in 1925 by the eminent Dutch microbiologist in Delft, A J Kluyver (8, 9), and since continuously espoused in this country by his disciple and former student and associate, C B van Niel, one finds it possible to discover a certain *uniformity* throughout the field of mold metabolism The very numerous different principal metabolic activities of molds can be looked upon as manifestations of a few main types of metabolic activity The great majority of them can be considered merely as extensions of the preceding ones so that gradually a series is built up, with comparatively simple examples on one end compounding successively to extreme complexity on the other Ramifications branch off the main series, to account for the extreme diversity of metabolic types encountered Viewed in this way, one perceives, in essence, what possibly might be considered as an evolutionary development from simple to complex metabolism, especially since in many cases the logical intermediate steps in the development of the series are known Or maybe (more likely!) the simple are derived from the complex through successive loss of function or of enzyme systems No argument is made that the schemes to be presented are evolutionary The main value of this idea is that is provides a

foundation on which the principles of mold metabolism can be resolved into orderliness

It may be emphasized at this point that this discussion deals with the biochemical origin in molds of the main kinds of organic compounds generally known to be formed by pure cultures of the organisms growing on media of relatively high carbohydrate content Commonly they are referred to as "waste" or "excretion" products, though, as seen later, this need not be the correct interpretation Not included here is the mechanism of formation of vital components of the cell, i e , protoplasm, nor the mechanism by which CO_2 originates from sugar except where it has a bearing on other points under consideration. Under scrutiny here is the third of the three main fates of carbohydrate carbon, namely, metabolic products, the other two being cell material and CO_2 Only generalized concepts will be given, the various processes are treated in detail in numerous papers and treatises devoted to the individual processes

The object of the following schematic presentation is to bring out the logical relations which exist among the main products of mold carbohydrate metabolism, and, where possible, to indicate that many of them have intermediary synthetic steps in common In several instances a product accumulated by one organism represents a simple further transformation of a product formed characteristically by another mold In the latter, the substance accumulates, due to inability of the fungus to effect further conversion quickly The further conversion is effected in the former, hence the first substance does not accumulate, but a second one does In this way it is possible to visualize a common metabolic channel for most fungi, any one differing from others by its ability to carry out one or more additional simple, single step reactions

It is to be expected that in the more complex of metabolic systems, evidence for intermediates and transformations common to the simpler metabolic system would exist Wherever investigated, this has been found to be true

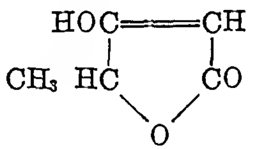
Not only can one find this kind of stepwise metabolic sequence among closely related organisms, but there are numerous instances of the same or similar sequences between distantly related organisms Seemingly this points to a certain unity of biochemical actions throughout the whole of this group of organisms, a conclusion entirely compatible with Kluyver's generalized concept There appears to be no general pattern relating taxonomy to biochemical potentialities within the fungi, and this could mean that metabolic offshoots evolved independently of structure On this basis it is therefore in agreement with expectations that the same major types of biochemical activity are found to occur among widely unrelated groups of fungi

Thus, most of the Mucorales, and certain penicillia and aspergilli have a preponderant C_2 metabolism, producing from hexose ethanol, acetic acid, with or without oxalic acid [5] Others carry this C_2 stage through the C_4 dicarboxylic acid stage only, producing mainly fumaric, malic and succinic acids, and this is

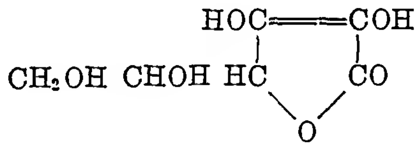
[5] Indeed, available criteria make it likely that in filamentous fungi as a whole the dominant carbohydrate metabolism centers about primary formation of C_2 split products

typical of the genera *Rhizopus* and *Fusarium* also. C_2 fractions can always be found as intermediates in these processes. In still other mucors, aspergilli and penicillia, as well as other fungi, these C_2 and C_4 compounds are used as precursors of citric acid, which accumulates in large quantities. Yet the C_2 and C_4 intermediates can usually be detected in the medium, accompanying, in small amounts, the major end product, namely citric acid. Though little experimental evidence is available, it is likely that in certain other aspergilli, *Aspergillus itaconicus*, for example, the citric acid or rather its equilibrium product, aconitic acid, functions only as an intermediate, not accumulating but being further converted through a further simple step into itaconic acid by decarboxylation. This is the most logical account of the accumulation of itaconic acid by these organisms.

One is also reminded by this line of reasoning of the simple chemical relations between the 5-membered ring acids produced by *Penicillium charlesii* as revealed by the Raistrick school. These are γ-methyltetronic acid, carolinic acid, carlic acid and carlosic acid. In addition, ethylcarolic acid (terrestric acid) is formed by *Penicillium terrestre*, and the latest stage in the picture as it exists today is the synthesis of ascorbic acid by *Aspergillus niger* (6). All these compounds are differently substituted tetronic acid derivatives, the relation of ascorbic acid to tetronic being as follows:



γ-methyltetronic acid



ascorbic acid

In these cases, it appears that the metabolism is common, the organisms differing in their ability to carry out the final simple transformations. In the case of *P charlesii* the synthetic sequence is also carried out by a single organism, but the other species mentioned can carry out modifications of this sequence.

Analogous systems exemplifying this principle may be found in the formation of 3,5-dihydroxyphthalic acid and three other derivative acids (C_{10}) by *Penicillium brevi-compactum*, and in the formation of different chemically homologous anthraquinone pigments by different species of *Helminthosporium* (5).

The main theme of the following scheme centers around the formation of split products from carbohydrate, and the type and fate of those split products. Based on this idea the following groupings are possible.

(A) No split products formed during sugar (hexose) utilization. In all these reactions the carbon skeleton of the carbohydrate is left intact.

(1) Gluconic acid and other sugar acids from aldosugars by oxidation of the aldehyde group, including galactonic acid, mannonic acid, xylonic acid, arabonic acid, etc.

(2) Uronic acids, in which the primary alcohol group is converted to a carboxyl: glucuronic acid, etc.

(3) Dicarboxylic sugar acids resulting from oxidation of both the aldehyde and the primary alcohol carbons to carboxyls: saccharic acid, mucic acid, etc.

These processes may be represented as follows:

$$\begin{array}{lll} & \nearrow & CH_2OH(CHOH)_4COOH \text{ (gluconic acid)} \rightarrow \\ CH_2OH(CHOH)_4CHO \text{ (glucose)} & \rightarrow & COOH(CHOH)_4CHO \text{ (glucuronic acid)} \rightarrow \\ & \searrow & COOH(CHOH)_4COOH \text{ (saccharic acid)} \leftarrow \end{array}$$

(B) Split products formed during sugar utilization

In common with that of all other living systems, the dissimilation of hexose sugars by molds follows uniformly the well-known mechanism of sugar breakdown through the triose or pyruvic acid stage, here referred to as C_3 compounds. And, as in the case of bacterial metabolism where many and diverse metabolic end products are encountered, the nature of the end products depends on how molds dispose of the intermediate C_3 compounds, this in part being a function of the enzyme makeup of any particular organism. In view of its easy transition from C_3 compounds and its extremely important metabolic significance, acetaldehyde (C_2) may also be considered with the C_3 compounds for the moment. Just as in the case of all other living systems, C_3 and C_2 compounds are the key intermediates in the formation of almost all mold metabolic products. The other main influence on the disposition of the C_3 and C_2 compounds is the degree of anaerobiosis *vs.* aerobiosis, or in effect, the availability of oxygen.

Since the origin of C_3 and C_2 compounds lies in dismutation reactions independent of oxygen, the ultimate metabolic products may be considered to have passed through two stages of metabolism, the initial stages, anaerobic or *fermentative*, and the final, either a continuation of anaerobic reactions or the participation of aerobic reactions, depending on the compound. Often, for the second stage, a mold may possess enzymes capable of effecting both anaerobic and aerobic transformations. In such cases, and similar to most normal cells, the availability of oxygen generally suppresses the so called anaerobic reactions, though not always, *viz.*, lactic acid formation by certain of the Mucorales.

In addition to the above aspects of the fate of the C_3 and C_2 intermediates are two other main features:

These fragments are transformed in various ways without changes in the carbon chain and are left finally still as C_3 and C_2 compounds.

The fragments undergo condensation reactions leading to the accumulation of more complex compounds of higher molecular weight.

The condensation may be pure, involving either only C_3 or C_2 compounds, or, as is likely in some cases, may be mixed, in which C_3 and C_2 compounds may

combine with the other or with condensation products of the other The condensations may be simple, involving only two or three molecules, or it may be highly multiple, leading to very complex high molecular weight compounds

I C_3 split products

(a) Simple conversion lactic acid, glycerol, pyruvic acid

(b) Condensation

Two molecules→ Kojic acid[6] (3), hexose sugars, single ring compounds

Many molecules→Complex ring compounds, pigments, including anthraquinones Many compounds isolated by Raistrick school

Of the condensation reactions only kojic acid and hexose sugars have experimental evidence in their support On the basis of the ring synthesis in kojic acid, the idea is, by analogy, extended to include polycyclic compounds, although no evidence whatsoever is available on the synthesis of these compounds It should be emphasized that kojic acid is a C_6 ring, whereas many benzene ring type compounds are known to accumulate in mold cultures

II C_2 split products

(a) Simple conversion acetaldehyde, ethanol, acetic acid, ethylacetate, oxalic acid

(b) Condensation

Two molecules → (C_4) succinic acid, malic acid, fumaric acid, oxalacetic acid

$$C_4 + C_3 \rightarrow C_7 \xrightarrow{-CO_2} \text{aconitic acid}$$

or

$$C_4 + C_2 \longrightarrow \text{aconitic acid}$$

$$\text{aconitic acid} \rightleftharpoons \text{citric acid} \xrightarrow{-CO_2} \text{itaconic acid}$$

8-9 molecules C_2 → higher fatty acids stearic, oleic, palmitic, etc

$$\left.\begin{array}{c}\text{Several molecules } C_2\\ +\\ \text{Several molecules } C_3\end{array}\right\} \rightarrow \text{Complex high molecular weight pigments, and other synthetic compounds}$$

Worthy of mention in connection with the condensation reaction in this section is that the products of primary condensation, which are excreted and accumulate in cultures of some organisms, may, in other organisms, participate in further condensation reactions leading to larger molecular weight compounds In the

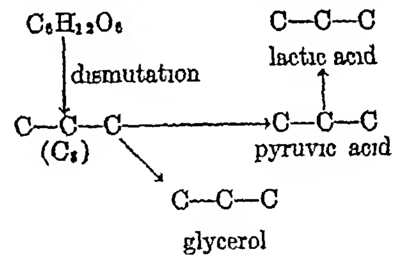
[6] Kojic acid is also claimed to be formed through dehydration and ring closure, producing the γ-pyrone direct from the hexose molecule without primary split products being formed (2, 11)

above listing, citric acid originates from the condensation of a primary condensation product, C_4, and a C_1 or C_2 compound Similarly, many of the complex pigments and anthraquinones could be interpreted as arising from secondary condensations of the primary condensation rings, namely, simple unicyclic rings Oxidations, alkyl chain synthesis, etc , all are involved in the building of the final molecule A scheme based on such ideas has been presented by Tatum (14) to account for the origin of the numerous and diverse complex substances isolated from molds and described by the Raistrick school Again, this scheme is based on chemical logic, there being, unfortunately, absolutely no experimental evidence available by which to be guided Nevertheless, this kind of inductive correlation is of great value in coördinating what otherwise might appear to be a confusing mixture of different chemical substances The value of Tatum's sequence is that it affords a credible explanation as to how the simpler of Raistrick's substances, isolated from certain fungi, may be further converted by additional condensations, substituent incorporations, oxidations, etc , into the more complex structures isolated from other fungi An analogy to the origin of citric-aconitic acids is apparent

It will be noted that there are two mechanisms for synthesis of C_4 compounds, namely, $C_2 + C_2$ and $C_3 + C_1$ Probably another mechanism, yet to be demonstrated in molds, is their origin from α-ketoglutaric acid (C_5) by decarboxylation

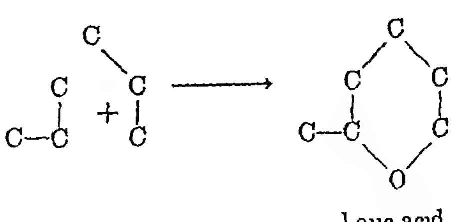
$$C_5 \xrightarrow{-CO_2} C_4$$

Carbon Skeleton Transformations Involving C_3 *Compounds*

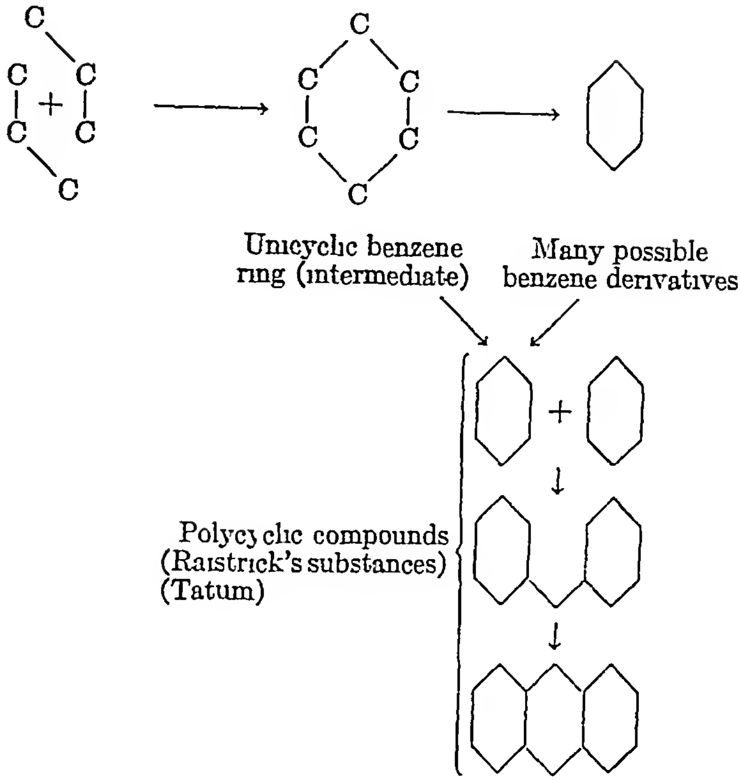
(a) 

(b) C—C—C + CO_2 ⟶ C—C—C—C oxalacetic acid

(c) C—C—C + C—C—C → C—C—C—C—C—C → (C—C—C—C—C—C)$_n$
hexose polysaccharide

(d) 

(e)



Carbon Skeleton Transformations Involving C_2 Compounds

(f)

(C_3) C—C—C $\xrightarrow{-CO_2}$ C—C (C_2) ⟶ C—C acetaldehyde

C—C oxalic acid
↑
C—C acetic acid
↑
C—C acetaldehyde
↓
C—C ethanol

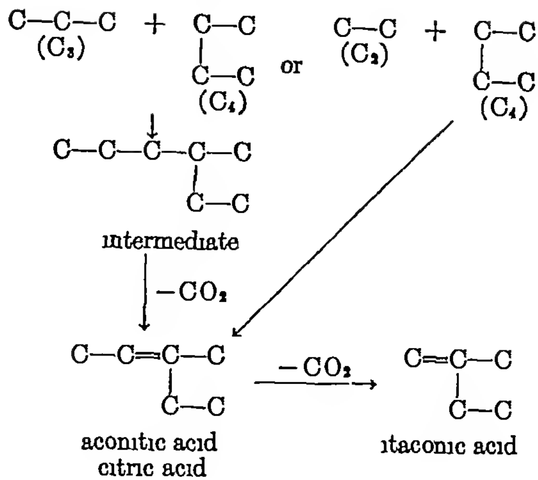
[C—C + OOC—C ethyl acetate]

(g) C—C + C—C → C—C—C—C succinic acid
↓↑
C—C—C—C fumaric acid
↓↑
C—C—C—C malic acid
↓↑
C—C—C—C oxalacetic acid

(h) $(C—C)_8$ → C_{16} straight chain, palmitic acid

(i) $(C—C)_9$ → C_{18} stearic, oleic, linoleic acids

(j)



(k)

RESERVE STORAGE MATERIALS?

It is obvious that throughout the infinite variety of molds studied, on account of their diversified enzyme makeups, the products of overflow or shunt metabolism will be equally diverse Not unexpected is the finding that some are water insoluble, or are non-diffusible through the cell membranes, and, consequently, are deposited within cells and accumulate there in substantial quantities, amounting in some cases up to 50 per cent of the total weight of the cell material Considered in this light, metabolically speaking, there are two main types of metabolic shunt products, those soluble and diffusible in water, and those which are not Fats, polysaccharides, complex pigments, and compounds of the Raistrick type which are deposited in and accumulate in mycelium are insoluble shunt products whereas organic acids, alcohols, pigments and other low molecular weight compounds are soluble shunt products

There is no more reason for considering fat and polysaccharide depositions as reserve or storage products, than the carbohydrates and organic acids formed and which accumulate outside the cells One might even include the complex benzenoid pigments found by the Raistrick school to comprise 30 to 40 per cent of the cell material The principal arguments advanced in support of the "reserve material" hypothesis are two they are laid down during luxury carbohydrate nutrition of the organism, and they disappear, by cellular oxidation, during starvation conditions, supposedly serving as a source of energy and carbon

The first argument is equally true of all products, including organic acids, accumulated by molds either within or outside the cell. There is ample evidence also that the second argument is not peculiar to the intracellular accumulations The observation is a rather general one that extracellular accumulations also tend to be utilized by the organism after exhaustion of the primary energy source, namely carbohydrate Yields of most, if not all, organic acids tend to fall after reaching a maximum coinciding with depletion of the sugar Just as fat dis-

appearance is slow and gradual, so is the attack on the initially accumulated soluble organic acids and carbohydrates

In the final analysis this is simply a reflection of the ability stated above of fungi eventually to oxidize completely the original available substrate to CO_2 and H_2O, besides synthesize cell material So long as the organism is surfeited with easily consumed carbohydrate, the attack on shunt byproducts is deferred Relieved of their saturation by the preferentially decomposed sugar, the dismutation enzymes now proceed with the slower degradation of the initially formed products of deranged metabolism

Actually, the rate at which accumulated fats are attacked by the mold is so slow that it is difficult to see how they could be of value to the organism as a "reserve product" No evidence is available, but it seems possible that intracellular accumulations of the complex compounds, pigments, benzenoid compounds, etc, also might be further attacked and slowly consumed, provided an abundance of oxygen is available Functionally speaking, these compounds could very well be considered in a class with other mold products Thus, extracellular accumulations of metabolic products would have to be considered as storage or reserve products by the same interpretation that intracellular materials are Such a conclusion seems untenable

More acceptable are the ideas presented above which lead to the conclusion that all such compounds happen to be subject to degradation by the mold irrespective of their location A water soluble, diffusible compound is by ordinary concepts just as available to the cell as insoluble fat in a vacuole The attack (and consumption) of these accumulated products probably never begins until the organism exhausts its more easily attacked and preferred energy source—carbohydrate

This article could not be complete without my pointing out that I commenced writing it almost to the day ten years after I came under the tutelage of Professor S A Waksman at New Brunswick, New Jersey He is responsible for this article, for he first introduced me to this general subject, acquainted me with its lure and potentialities, urged me to make it my following, and has since, by virtue of his sagacity, zeal and intimate personal friendship and counsel continuously through these years, unwittingly shaped my scientific outlook and extended my scope For all this, and with appreciation, I dedicate to him this article on one of his favorite subjects

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THE THERMOPHILIC MICROÖRGANISMS

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The phenomenon of thermobiosis, including both survival and growth at elevated temperatures, has attracted the attention of biologists sporadically for almost two centuries. The earliest recorded observation was that of Sonnerat (246) in 1774 in which fish were described living at a temperature of 69 R. (*ca.* 82 C) in a thermal pool on the Island of Luzon. This observation received a published letter of confirmation from the Commissioner of the French Navy (206). Fifty years elapsed before a revived interest revealed algae growing in thermal springs (*cf.* 284). Soon fishes, molluscs, arthropods, worms (76, 134), molds (160, 201, 97), and members of all the classes of algae (240, 51, 107, 161, 203, 84, 26, 76, 45, 242) were found growing at temperatures of 60 to 98 C. The extensive literature before 1860 was of a purely descriptive nature without intent of explaining such a peculiar phenomenon (190).

With the development of the field of bacteriology came the description of many more fungal species from thermal springs. Although the work continued in a descriptive vein, observations were presented more completely, and theories of the origin and nature of these unique organisms became numerous.

Although the initial discovery of a thermophilic bacterium is attributed generally to Miquel (175) in 1879, the early descriptions of Long (134), Hooker (117), and Brewer (26) indicate that among their thermophilic "Confervae" were representatives of the *Chlamydobacteriales*. A complete bibliography of early workers (prior to 1927) who have described thermophilic rods, both sporulating and non-sporulating, is given by Robertson (225). A study of the papers cited in this source and the great number of publications in the last twenty-five years dealing with the spoilage of food, particularly processed foods, by thermophilic microörganisms indicates that the bacilli are the forms most commonly encountered. Among the cocci, we find sarcinae (2, 99), staphylococci (51, 232, 257), and streptococci (264, 98, 199) described as thermophilic, although it is more likely that their capacity was one of resistance rather than active growth at elevated temperatures. Specific reference to the higher bacteria has been made largely to the actinomyces (97, 139, 258, 259, 178, 179, 234, 261, 96, 29, 171, 172, 238, 208, 19, 146, 126, 173, 235). Two thermophilic spirochaetes

(41, 28) have also been noted Higher fungi of thermophilic character have been described (259, 238, 149, 171) and a summary has been presented by Noack (188)

The ubiquitous nature of the thermophiles is attested to by the great variety of sources from which they have been isolated—from freshly fallen snow (99, 215), to the sands of the Sahara Desert (187) They have been found to occur in the air (175, 234), the soil of temperate (97, 163, 215, 193, 139, 96, 23, 237, 258, 259, 234) and tropical (150, 97) regions, salt (163) and fresh water, both cold (175, 215, 193, 170, 44, 257) and thermal (45, 163, 137, 201, 178, 179, 79, 93, 94, 242, 260, 18), on grains and foods of all varieties (264, 215, 193, 154, 205, 13, 236, 133), in raw and pasteurized milk (215, 234, 156, 83, 275, 232, 29), in the feces of all domestic animals and man (175, 163, 215, 258, 259, 234, 261, 29, 8, 69), birds and a variety of fishes and frogs (215), and in stored vegetable material (171, 163, 164, 24, 13, 238, 133, 14, 266, 33, 103) Older reviews may be consulted for the detailed account of the occurrence of these organisms in water (8, 181), soil (149, 150, 171, 146), milk (250, 225, 208, 135), and food (2, 39, 165)

Despite the fact that climatic conditions apparently have no influence on the distribution of thermophilic bacteria (176), several instances of soils free from thermophiles are of note Migula (174) states that uncultivated soils may be entirely free from thermophiles, and Tsiklinsky (262) was unable to find thermophilic microörganisms in the samples of soil collected from antarctic regions by the Polar Expedition of Charcot Nevertheless, there seems little doubt that the thermophilic bacteria are natural soil inhabitants and can be isolated from any material which has come in contact with soil

DEFINITION OF TERMS AND CHARACTERISTICS OF THERMOPHILIC MICROÖRGANISMS

Each worker who isolated one of the so-called "thermophiles" gave it a species designation or number, but failed to provide sufficient data to make possible a comparison among organisms Even today this vague group of bacteria termed "thermophiles" has few adequately defined species

Prior to the discovery of thermophilic bacteria, mention was made only of the ability of an organism to live at high temperatures When bacteria were found with this same thermal tolerance, an effort was made to define the temperature range for growth Although this range had considerable latitude, any bacterium which grew at elevated temperatures was considered a "thermophile", regardless of its optimum or minimum temperatures for growth The maximum temperature tolerated generally lay between 50 and 90 C, and the optimum range for the apparent maximum population yield was slightly below the maximum temperature for growth (2) Occasional reference was made to "strict" or "obligate" thermophiles which failed to grow at lower temperatures

In 1898 Schillinger (237) made great point of the unsatisfactory condition of the literature on the thermophiles and stressed a change in nomenclature He

proposed the term "thermotolerant" for bacteria which grew at both high and low temperatures and reserved the term "thermophilic" for bacteria which grew at high temperatures but not at body temperature, although he questioned the existence of the latter group Sames (234) also stressed the importance of this distinction

By 1907 Miehe (171) had completed his most extensive comparative study of all available cultures of thermophilic species The purpose was not only to establish synonymity among the members of this group, but also to make a comparison of the general properties of this group and other bacteria Although Miehe points out a definite compactness of the thermophiles as a group, he cannot deny the difficulty involved in fixing the limits of the temperature ranges for the various groups of bacteria He arrived at the following nomenclature *Thermophiles* organisms with a temperature minimum of about room temperature (25 C), *Orthothermophiles* thermophiles with a temperature maximum above the temperature of protein coagulation (60–70 C), *Thermotolerants* organisms with a temperature maximum of 50–55 C, but which also grew well at room temperature

Little use was made of the criteria set up by Miehe, and Schillinger's designations were employed more frequently Bergey (19) divides thermophiles into two groups *True Thermophiles*, which show optimum growth at 60–70 C and no growth, or only very feeble growth, below 40 to 45 C, *Facultative thermophiles*, which develop at room temperature, about 20 C, and have their optimum temperature at about 50 C, and their maximum at about 60 C

Morrison and Tanner (182) believe that the difference in temperature ranges for the various groups of bacteria can be expressed best by a system of classification based upon the optimum temperature for growth, rather than the temperature limits for growth Thus, they believed that the organisms that grew at elevated temperatures could be placed in a "homogeneous" group, consisting of the following types *Strict Thermophiles*, optimum temperature above 55 C, *Facultative Thermophiles*, optimum temperature 50–55 C, *Thermotolerant Bacteria*, optimum temperature 40–50 C Cameron and Esty (38) have also used the term *facultative thermophile*, but without reference to optimum temperature, the interest being merely in distinguishing between *obligate thermophiles* (grow at 55, but not at 37 C) and *facultative thermophiles* (grow at 55 and 37 C) These terms have since been used commonly in describing organisms isolated from milk (e g , 71, 208) It was found necessary also to coin new words to describe the heat-resistant flora of milk, e g , *thermoduric mesophiles* (71, 225–8) which exhibit optimum growth between 20 and 37 C and survive pasteurization in large numbers Molds generally have been considered thermophilic if their optimum temperature for growth is between 40 and 50 C (188)

The most recent designations, reviving the early descriptive terms of Fischer (82) and the animal physiologists, are those of Imsenecki and Solnzeva (129) "True thermophiles" are those species whose optimal range of growth lies between 55 and 60 C They may be divided into two groups "Stenothermal thermophiles", develop at 60 C but show no growth after many days at 28–30

C, "Eurithermal thermophiles", develop at 60 C, and show slight to abundant growth at 28–30 C

The multiple connotations of the terms employed to describe thermophiles has led to much misinterpretation The significance of such descriptive terms in a classification of thermophiles is questionable As in other taxonomic studies, we must concede the impossibility of dividing the thermophiles arbitrarily into a number of groups with specific temperature ranges From a survey of past work, it would seem adequate to designate bacteria with an optimum temperature for growth between 50 and 60 C as thermophiles, and, if necessary, to use the terms of Imsenecki and Solnzeva for defining the magnitude of the temperature range for growth

The question of synonymity of species names was apparent early in the study of thermophilic bacteria Inadequate description of the characteristics of an organism, coupled with failure to maintain type cultures for comparative purposes, has led to a confusion of duplicate names Bruini (29), Miehe (171), Morrison and Tanner (181), and Prickett (208) have contested the identity of many of the species presented in the literature However, on the philosophy that less confusion is caused by an organism having two names than by two organisms having the same name, new names have continued to appear in the literature For example, Beaver (11) has described 32 new species of thermophilic spore-forming rods with very descriptive species names Species distinctions were based upon such insignificant differences as the relative time required to effect a particular biochemical change, the abundance of growth on a particular medium, and so on The strikingly similar morphology and physiology of the aerobic and facultative thermophiles (38, 181, 182) has led several workers to the assumption that all the spore-forming organisms are variants of a few type species

The Committee on Classification of the Society of American Bacteriologists under Bergey (20) has attempted to standardize the terminology of this group, and the subsequent revision by Chester (21) presents descriptions of 21 species of the Thermophilic Group, Family *Bacillaceae* The scheme is far from satisfactory, but is serviceable in that it presents in convenient form a portion of our knowledge of this type of organism The problem of segregating the assorted information in the literature is discussed by Prickett (208) Our present classification of thermophilic bacteria, based upon slight differences in spore size and location, ephemeral pigments, etc , of inadequately studied cultures, assists very little in the identification of a newly isolated organism

Common morphological characteristics of aerobic and facultative spore-forming thermophiles, described up to 1928, have been pointed out in detail by Prickett (208) In addition to the similarities cited, all these thermophiles, in youth, are gram positive, with the possible exception of *Bacillus stearothermophilus* (66) It is interesting to note that of all aerobic and facultative spore-forming organisms of this group there is only one species listed which produces gas in its utilization of carbohydrate materials However, the original, and only, description of this organism, *Bacillus thermoamylolyticus* (52), indicates that the investigator was working with a mixed culture

Equally interesting is the observation that the literature reveals few cellulolytic thermophiles with adequate description for comparative purposes. Despite the early interest of MacFadyen and Blaxall (164) and the detailed studies by Omelianski (191), Pringsheim (211-3), Kroulik (147), Tetrault (254) and others of the University of Wisconsin group (*cf.* 186, 189), and the recent Russian workers, little knowledge of the thermophilic cellulose-digesting organisms in pure culture has been gained.

Superficial studies and ill-defined species have resulted from the industrial interest in the use of these organisms for the disposal of cellulose wastes. However, the tremendous difficulty in establishing unequivocally the purity of cultures of thermophilic cellulose bacteria is responsible largely for the confusion in this group of thermophiles. The purity of aerobic and facultative cultures capable of hydrolyzing cellulose (268, 241, 140, 202, 245, 12, 147, 53, 283, 254, 255, 87, 132) long was questioned (214) and still is to be contested. The symbiotic nature of most of the cellulose fermentations prevents us from attributing all of the products to thermophilic activity. The wide variety of gases, acids, and neutral volatile materials is illustrated well by the summary of Buswell and Hatfield (34).

Cultures which are active in digesting cellulose when cultivated aerobically and anaerobically have been found frequently to include an anaerobic species and a facultative species. *Clostridium thermocellum*, described by Viljoen *et al.* (268), is thought now to be such an association. It is recognized also that obligate anaerobes (263) are composites of associated forms (244). Murray (185), however, has obtained pure cultures of aerobic and thermophilic cellulose-digesting bacteria. The inability of other workers to obtain aerobic growth in agar of thermophilic cellulose-splitters has been attributed to inadequate humidity. A saturated atmosphere was found to be necessary for optimum growth of these organisms.

The symbiotic nature of cellulose fermentation has been clarified recently by the Russian microbiologists (122, 127, 230, 120, 229, 231, 121). Anaerobic decomposition of cellulose by thermophiles has been resolved into two processes: the hydrolysis of cellulose, and the subsequent fermentation of hydrolytic products. In pure cultures of cellulolytic thermophiles most of the hydrolytic products (40 to 75% of the cellulose carbon as glucose) accumulate in the medium, and only a portion is fermented to yield carbon dioxide, hydrogen, and acetic, butyric, formic, and lactic acids. In mixed cultures, i.e., in symbiosis with other organisms, higher yields of acids and alcohols, and products, such as methane, not formed in pure culture fermentation, are obtained. The highly cellulolytic activity of the thermophiles thus effects an accumulation in the medium of a carbon compound which is readily fermentable by the concomitant organisms. The activity and characteristics of pure cultures of a number of cellulolytic thermophiles, such as *Bacillus cellulosaedissolvens* and *Clostridium ellipsosporogenes*, have been reported.

By definition, organisms other than the spore-formers also must be grouped with the thermophiles. Most notable of these are several of the lactic acid bacteria. Henneberg (114) and Orla-Jensen (195) have described lactic acid

bacteria with an optimum temperature in the region of 50 C However, Tsiklinsky (263), one of the outstanding investigators of thermobiosis, a few months before her death in 1921, isolated lactic acid bacteria with elevated optima, and with minima between 42 and 45 C *Lactobacillus thermophilus* (7, 47) of Orla-Jensen's sub-genus *Thermobacterium* is the only well-studied organism which has a temperature optimum above 50 C (optimum 50–62 8 C, minimum 30 C, maximum 65 C) Other non-sporulating rods and cocci, which have been considered to be thermophiles, require more study before they can be placed in this category Cocci which have been called thermophilic (257, 99, 199, 195, 264, 232) undoubtedly multiply at temperatures in excess of 50 C, but find their optimum far below this point The cocci and non-spore-forming rods presented in the early descriptions of van Tieghem (264), must be discounted *Denitrobacterium thermophilum* of Ambrož (3) from all indications was a thermophile There are probably also thermophilic filamentous bacteria with optima above 50 C, but a summary must await a more detailed study of these forms

Among the anaerobic spore-forming thermophiles we find greater confusion than among the aerobes The generic name *Clostridium* has been applied indiscriminately to facultative anaerobes (154, 205) Obligate anaerobes (236, 187, 265, 261) and facultative anaerobes (193, 18, 8) have been described briefly on a number of occasions, but given species names infrequently Confusion with regard to the oxygen requirement of some organisms has arisen as the result of the misconception (215, 238) that aerobic thermophiles with high temperature minima will grow at ordinary temperatures if cultivated anaerobically

The best defined spore-forming anaerobes of the thermophilic group are *Clostridium nigrificans* of Werkman and Weaver (280, 279) and *Clostridium thermosaccharolyticum* of McClung (166), both with a temperature optimum at 55 C or higher *Clostridium thermoacidophilum*, *Clostridium thermoaerogenes*, *Clostridium thermochainum*, and *Clostridium thermoputrificum* are obligate anaerobes described at length by Damon and Feirer (61) Werkman (279), however, was unable to confirm the anaerobic nature of *Clostridium thermoputrificum*, and McClung (166) failed to obtain the proper reactions when studying the other available cultures of Damon and Feirer

Non-sporeforming obligate anaerobes also may have representatives in the thermophilic group, for some grow very actively, if not optimally, at temperatures well above 50 C Several bacteroides-like organisms, originally isolated by Veillon (265) and later termed *Bacillus thermophilus* γ and β (277, 207), are grouped in Prevot's genus *Ristella*

Thermophiles, therefore, constitute a very heterogeneous group, if we include all organisms with an optimum temperature for growth above 50 C Their morphology, and even staining reactions, are varied Fundamental differences appear in their nutritional requirements and metabolic activities Most of the thermophiles will grow well on the common culture media, others require special nutrients The field of essential nutrilites for these organisms has been untouched

Although most investigators agree that thermophilic organisms are non-pathogenic, several accounts opposing this view are of note Bruini (29) injected whole cultures of thermophilic organisms into guinea pigs and attributed death of the animals to the toxic products formed by the thermophilic cells Ascione (6) described a thermophilic streptothrix which apparently produced a hemolytic toxin The validity of the results of these two workers is not beyond question Black and Tanner (22) have reviewed the subject of pathogenicity of thermophilic organisms

The greatest number of the thermophiles are facultative with regard to the oxygen tension under which they are capable of developing Despite much conflicting evidence in the literature, there are in addition, aerobic and anaerobic forms The literature indicates that the facultative organisms exhibit widely different oxygen tension requirements for optimum growth, i e, some grow best under aerobic conditions, while others are favored by an anaerobic environment

Most of the thermophilic bacteria are capable of producing spores, but the ability and degree of sporulation under a given set of conditions varies among cultures The great heat resistance of these spores is assumed generally The body of data on this subject will be discussed in a later section

The fate of various substrates shows considerable diversity Various thermophilic organisms are unable to utilize the simple mono-and di-saccharides (278–280), starch (94, 23, 208), and cellulose Apart from the mere utilization of carbohydrate material, fundamental differences in endproducts are noted when a carbohydrate is fermented Thermophilic organisms which attack carbohydrates are most important in the spoilage of foods, and consequently have received greatest attention Research workers of the National Canners Association and the American Can Company have divided these organisms into two groups and have given numerical, rather than species, designation to members of the groups with differences in cultural reactions, i e , flat sour organisms aerobic and facultative bacilli characterized by the production of acids (lactic, formic, acetic) but not gas, gas-forming (non-H_2S) anaerobes anaerobic bacilli which produce acid and large quantities of gases (CO_2 and H_2) from carbohydrates

For most thermophiles described in the literature it is impossible to determine the action on protein materials The universal use of gelatin and milk provides very little information Early workers (215) were led to conclude that the most important characteristic of thermophilic bacteria was their proteolytic activity Although some thermophiles have been described as highly proteolytic, such as *Bacillus delbrueckii* (12) and several non-cellulolytic bacteria isolated from manure (69), sewage (8), water (181), and milk (83), the activity of these organisms in symbiotic relationships in nature suggests that they are, at most, feebly capable of attacking native proteins (196) This has been found to be true in more recent work with pure cultures of food spoilage organisms, called hydrogen sulfide or "sulfur stinker" organisms (165, 279), and thermophiles from milk (71) Hydrogen sulfide and indole production are the only end-products of organic nitrogen metabolism studied Indole is apparently pro-

duced by a limited number of thermophilic organisms (181), hydrogen sulfide is not a common product even among the anaerobes Reduction of nitrate to nitrite is encountered frequently among the thermophiles (21), although reduction of nitrite is infrequently cited (233) *Clostridium thermosaccharolyticum* is unable to reduce nitrates, but can reduce nitrites (166)

The following types of thermophilic microörganisms have been described nitrogen fixing (210, 149), nitrosifying (40), denitrifying (80, 19, 3, 22, 243, 150, 8), sulfate reducing (77, 247, 248, 75), sulfur and sulfide oxidizing (179, 242, 58), iron (178), proteolytic (193, 150, 19, 80, 243, 196, 22), amylolytic (193, 23, 19, 52, 243, 22, 130, 123, 125, 128), lipolytic (150), and halophilic (28) A number of thermophiles are capable also of oxidizing phenol and various hydrocarbons (73, 74)

ORIGIN AND DISTRIBUTION OF THERMOPHILIC MICROÖRGANISMS

It is understandable that subsequent to the search for members of this apparently abnormal group, efforts were directed toward establishing their origin, an interpretation of their wide distribution and abundance, and the mechanism by which they are so resistant to heat

Conjectures as to origin have varied from the logical to the fantastic Thus, workers, like Rabinowitsch (215, 216), Schillinger (237), Tsiklinsky (258–260), Jancke (135), Miehe (171), and Imsenecki and Solnzeva (129) considered them variants of well-known strains of mesophilic bacteria, progressively more completely adapted to higher temperatures up to the final obligate stage, others (18, 163, 29) considered that they had become adapted even more gradually and more reversibly than connoted by the term "variant" Lieske (158) and Kluyver and Baars (143) believe that they are the result of spontaneous adaptation or mutation, occurring in one step Thermoresistant forms resulting from mutation have been found in various of the lower animals (16), and Ricket *et al* (224) have reported an hereditary shift in growth optimum from 36–37 C to 41–42 C for a lactic acid bacterium after treatment with KCl

Weed (276), and later Ambrož (2), suggested that thermophilic microörganisms may be "reminders of the thermophilic flora of earlier geological periods" —perhaps influenced by descriptions of forms like Renault's giant bacteria of the carboniferous age (223) Molisch (180) and Golikowa (99) also maintain that these organisms have arisen, not by adaptation from lower to higher temperatures, but rather that they have arisen at elevated temperatures and some have become adapted very slowly to lower temperatures Sames (234), de Kruyff (150), and Miehe (171) have indicated the tropics as the locus of the evolution of thermophilic microörganisms

Svante Arrhenius (5) discounts such an adaptation process on this planet, and considers the natural habitat of the thermophilic bacteria to be the planet Venus, where the average temperature is 47 to 50 C The organisms or their spores are believed by him to be propelled by the radiation pressure of the sun and to travel from Venus to Earth in a few days

In all probability the thermophilic microörganisms had their origin in some locality of tropical climate, and are found today in greatest numbers where elevated temperatures prevail. The role of the thermophilic bacteria in the economy of nature proved to be as puzzling as their origin. Temperatures between 50 and 60 C are not uncommon in tropical soils (105) and the activity of thermophilic forms in this environment is of great significance (150, 97). Their distribution in lesser numbers over the entire surface of the earth opened vast fields of study and speculation with regard to their existence in regions where the temperature seldom, if ever, reaches a maximum of 40 C.

The direct heating action of the sun in the temperate zone has been found (234, 97, 98, 188, 146) to be sufficient to permit multiplication of thermophiles in superficial top soil, mud puddles, and fallen vegetation. In addition, the processes of putrefaction and fermentation, effected by mesophilic organisms, provide adequate heat for active germination and growth of thermophiles (163, 29, 99). Attention was directed, therefore, toward spontaneous heating and combustion of hay and manure piles (171, 172, 150, 51). Maximum temperatures reached within piles of vegetation have been reported to lie between 60 and 90 C (171, 238, 133, 168, 116, 92, 69). Noack (188) is of the opinion that the activity of thermophilic microörganisms does not depend upon man's agricultural pursuits, and presents evidence that the temperature attained in a pile of fallen leaves 50 cm high is adequate for the development of thermophilic flora. The subject of spontaneous heating and combustion of hay has been reviewed extensively by Browne (27).

Other workers (99) believe that, generally, the above situations constitute exceptional conditions under which thermophiles can develop, but in the absence of these conditions, the thermophilic microörganisms continue their activity in a symbiotic relation with mesophilic organisms.

Bacteriologists who consider all thermophiles to be thermotolerant in varying degree have presented voluminous evidence (215, 216, 261, 29, 237, 2) that the thermophilic bacteria thrive in the alimentary canal of man and animals. However, the mere presence of thermophilic bacteria in the intestine of warm-blooded animals does not constitute proof that these organisms find here a suitable habitat for growth. The actual number of thermophilic bacteria has been shown to be small in the case of human feces (4, 22), and perhaps largest in the feces of cattle (22). The part played by these organisms in the decomposition of the intestinal contents is unknown, but discounted frequently as unimportant (99).

An interesting observation on the distribution of thermophiles was made by Mischustin (177) in a study which employed the thermophilic bacteria as an indicator of the "cultivatedness of soil". Thermophilic bacteria were found in insignificant numbers in virgin soil, but upon cultivation of such soil, thermophiles were introduced with the manure, and developed rapidly to great numbers. According to this observer, the number of thermophilic bacteria in soil is closely related to the intensity of manuring.

EXPLANATION OF HEAT RESISTANCE AND GROWTH AT ELEVATED TEMPERATURES

Explanations for the ability of thermophilic organisms to carry on normal life processes at elevated temperatures, incompatible with the usual forms of life, must be based upon random findings drawn from many of the biological sciences From the pioneer work of Miquel (175) to the early twentieth century (257, 99), the belief that thermophiles contained a peculiar type of protoplasm was the extent of speculation The high resistance of thermal algae was attributed by many workers to the presence of dissolved gases (111) and other constituents of the medium The relation of thermal sensitivity of microorganisms and the chemical composition of the medium has been reviewed adequately by Bělehrádek (16)

Davis (63) was unique in his statement that thermophilic forms are able to withstand unusually high temperatures because of their "low grade of protoplasmic organization" Attention, however, soon was deflected from the thermophilic to the mesophilic forms and focused upon the bacterial spore, the most commonly recognized example of thermal resistance von Esmarch (271) maintained the idea that spores are surrounded by a protective coating which insulates them by partially preventing the passage of heat This assumption was opposed by Lewith (157) and Virtanen (269), the latter, by calculation, estimated that the spore wall would have to be a million times more insulating than air in order to exert any protective effect Evidence, however, has been presented for a protective coating, either a capsule (104) or a coating of questionable secretory origin (267, 226, 282, 100, 101) Hückel (118) was able to isolate from several species of mesophiles a non-specific protective substance secreted by the cells which, when separated from the culture by filtration, imparted greater heat resistance to less resistant organisms While it may be conceded readily that certain materials do exert a protective effect on cells subjected to heat, such a protection in the case of actively metabolizing cells is difficult to conceive It may be noted, however, that many workers today assume a special role for the spore coat in heat resistance (153)

Before the time of Pasteur, Doyère (68) had demonstrated the effect of water on the thermal resistance of rotifers and tardigrades A correlation of water content and heat resistance has been noted since then to apply to the spores and vegetative cells of many of the lower plants and animals (59, 62, 198, 239, 226) The assumption, however, that vegetative cells have a higher moisture content than spores was disproved by the work of Virtanen and Pulkki (270) in which it was found that no such difference in water content existed Cramer (54) and Benecke (17) have described a hygroscopic cell wall of carbohydrate and fat-like material capable of retarding diffusion By comparing the rates of diffusion of a dye into spores and vegetative cells, Benecke provided evidence for his theory and suggested permeability as a controlling factor in thermal resistance A score of subsequent observations (226) have confirmed the relative impermeability of the spore, and occasional indications (31) have been found that the degree of permeability of the vegetative cell wall to water de-

termines the heat resistance Robertson (226) concludes, from a study of thermophiles in milk, that changes in the nature of the "cell-wall membrane", and changes involved through acclimatization processes may be instrumental in producing a cell with a low moisture content and consequently a higher thermal resistance Also with regard to the thermophilic bacteria, Hampil (104) suggests that vegetative cells of these organisms have a lower water content than cells of mesophiles Apparent differences in water content may be of significance in an analysis of heat resistance, but of equal importance is the increased surface effects at elevated temperatures

At the time such early hydration theories were proposed, the existence of bound water in biological systems was unknown And despite the emphasis on water of hydration by Gortner and other workers (102), its physiological significance is even today a matter of heated argument (30) Evidence has been presented to explain the resistance to low temperatures of certain plants, seeds, spores, bacteria, and animals (113) which apparently contain little water The work has been based on the fact that bound water has a lower freezing point than free water, by virtue of the strong forces binding it Although it is known that the tenacity with which some substances retain water of hydration is indicated also by the higher temperatures required to remove it, few published attempts have been made to relate this character of bound water to heat tolerance of bacteria (88) A high bound water content, as found in spores, has been interpreted as a protective mechanism against coagulation of cell proteins The alteration of proteins, or "irreversible protoplasmic changes", constitute a traditional theory of death of organisms at elevated temperatures (49), despite early demonstrations that the coagulation of proteins does not parallel the thermal death point of the organism (151) Conflicting views were held with regard to the factor responsible for preventing or decreasing the rate of protein coagulation Shaw (206) contended that in thermophiles the higher specific gravity of the protoplasm was responsible, while Williams (281) and others (19) attribute the observed effect to the low mineral or ash content of resistant forms Very concrete evidence recently has been provided by the elemental analyses of Curran *et al* (55) in which the calcium content of spores was found to be considerably higher than for corresponding vegetative cells The authors note that the high calcium content may be related to the ability to bind water and to heat resistance Thermophilic bacteria have not been considered as yet for bound water studies

The observation that usually both death of microörganisms and inactivation of enzymes by heat proceed in logarithmic order, i e , a first order reaction, has led to the frequent association of death by heat and the thermolability of enzymes Common, also, is the assumption that heat resistant forms possess peculiar enzyme systems Virtanen (269) proposed that a firmer combination of enzyme and cell protein is responsible for increased thermal resistance Feirer (80) has claimed that the enzymes, catalase and diastase, of some soil thermophiles are active at temperatures where the enzymes of mesophiles are destroyed (104) Multiplicity of enzymes or a stronger enzyme-protein associ-

ation have been suggested by Rettger and his students (42, 72) to account for increased resistance The minimum temperature for destruction of catalase, indophenol oxidase, and succino-dehydrogenase, compared statistically with the maximum growth temperature of a bacterium, was claimed by these workers to show good agreement for the mesophiles and thermophiles studied A high degree of correlation was apparent for mesophilic cultures, but was questionable for thermophilic cultures Using nine strains of thermophilic bacteria with a weighted mean maximum growth temperature of 76 C, they found the minimum temperature for destruction of indophenol oxidase to be 65 C, of catalase, 67 C, and of succino-dehydrogenase, 59 C Rahn and Schroeder (222), however, denied the possibility of concluding from the work of Rettger *et al* that enzymes exhibit such behavior in normal living cells The use of resting cell preparations is considered faulty technique in that it provides an abnormal static condition, as opposed to the normal dynamic capacity of a growing organism to produce new enzyme molecules to replace those deteriorated Thus, the results of heat inactivation of enzymes, determined in resting cell preparations, and maximum growth temperature, determined in a complete medium, are not comparable Rahn and Schroeder (222), using *Bacillus cereus*, tested the data of Rettger by examining a suspension of cells in phosphate buffer for viability and enzyme activity as a function of temperature and time Invariably, enormous decrease in number of viable cells was accompanied by only slight decrease in activity of catalase and succinic dehydrogenase, the only two enzymes studied

Rahn (218) attributed death of bacteria by heat to endogenous catabolism, destruction of enzymes, or the inactivation of genes The logarithmic order of death of bacteria (48, 274) indicates that death must be due to the destruction of a single molecule Therefore, an explanation of death based upon the heat inactivation of enzymes (131) is untenable The general observation that the enzymes of bacteria function at temperatures above the maximum temperature for growth was found valid when extended to yeast (221) and bacterial (67) fermentations, to some of the respiratory enzymes of mesophiles (222), and to the complete respiratory system and its enzymic components in thermophilic bacteria (89) The inactivation of one of the heat labile enzymes involved in the very obscure synthetic reactions may satisfy the mathematical considerations of Rahn However, such an interpretation of the growth mechanism does not provide the only alternative Multiplicative reproduction, the bacteriologists' usual criterion of life, is not the inevitable sequel to growth Growth has occasionally been observed to continue after the faculty of division was lost The inactivation of a single gene essential to the reproductive mechanism, thus producing a sterile mutant (or "lethal mutant", as used by Jordan, 136), is consistent with the logarithmic order of death by heat and is maintained by Rahn (217, 219, 220) as the explanation of "death" of bacteria Such an explanation of death implies for thermophilic bacteria a heat stable genetic structure, in addition to the possibility of unique enzyme complexes

Tolerance of high temperatures has often been associated with the nature of

the lipids An inverse correlation between the melting point of the fat of an animal and the temperature at which the animal lives has been long recognized (115) The analysis by Leathes and Raper (155) and the extensive review by Bělehrádek (15) contain evidence that the protoplasmic and reserve fats and constituent fatty acids of animals and plants living at relatively low temperatures are more fluid, i e , less saturated, than the fats of animals and plants living at higher temperatures Leathes and Raper, in an attempt to explain the observed distribution of saturated and unsaturated fatty acids in nature, advanced an hypothesis based upon the usual theory of fat synthesis, namely the formation of long carbon chains with unsaturated linkages and subsequent saturation by reduction They maintain that the condensation reaction proceeds readily at low temperatures, giving rise to unsaturated fatty acids, but the reduction processes require a higher temperature Temperature, however, is not the only factor responsible for the saturation of fatty acids Terroine *et al* (252, 253) have verified, in part, the assumption of Leathes and Raper In a study of *Aspergillus niger* and the timothy-grass bacillus over a temperature range of 14 to 38 C, they observed a greater utilization of the potential energy of the medium at higher temperatures and the occurrence of more saturated fatty acids, both total and phosphatide, at higher temperatures Such data may be interpreted as indicating that the saturation of fatty acids by reduction succeeded the condensation reaction and involved an additional expenditure of energy Pearson and Raper (200) have also studied the total fatty acids of *Aspergillus niger* and *Rhizopus nigricans* over a narrower temperature range and demonstrated the influence of temperature on the saturation of the fatty acids formed The assumptions on which the general hypothesis was based are unproved, however, and the data in accord with the hypothesis are very scant Nevertheless, in the cases studied, it is clear that the temperature at which fats are formed is one factor which influences the degree of saturation of the lipids

Heilbrunn (112) and Bělehrádek (15) suggested that the melting point of the protoplasmic lipids determine the heat resistance of an organism Despite the intriguing aspects of this suggestion, the literature is strikingly devoid of experimental data Gaughran (89, 90), in a preliminary study of *Bacillus subtilis* and a stenothermophilic thermophile, found that for *B subtilis* the total lipid and its constituent acetone-soluble fat and phospholipid fractions decrease in quantity and degree of unsaturation as the temperature of cultivation is raised above the optimum, while the lipids of the stenothermophilic bacillus are strikingly constant both in quantity and degree of unsaturation This point will be discussed in the consideration of growth of thermophiles at low temperatures

Indirect evidence has been made the basis for many theories offered in explanation of thermal resistance and thermal requirement Experimental difficulties here are numerous, but not of such magnitude as to account for the deficiency of experimental data on this fundamental problem

It seems unnecessary to assume a unique protoplasm for the thermophiles or to conjecture about the mechanism by which the protoplasm resists "irre-

versible changes" As physical and chemical data on enzymes, proteins, bound water, permeability, surface phenomena, etc, accumulate, we may find differences between thermophiles and mesophiles "Irreversible changes", occurring in all cells, proceed at a correspondingly greater rate in thermophiles growing at elevated temperatures Even at their optimum temperature for growth, many thermophilic bacteria show an extremely high death rate Therefore, it is most probable that growth of thermophiles is not merely passive resistance to the unfavorable effects of high temperature, but rather may be attributed to their tremendous capacity of replacing compounds destroyed by heat The rate of destruction is not significant, if the rate of replacement is greater This situation is reflected clearly in the population curves of the thermophilic bacilli The duration of time for which thermophilic cells can maintain such an intense metabolic process is limited, and consequently their death rate is exceedingly high The mechanism of such a balance is, of course, open to conjecture Porter (204) postulated that the thermophilic cell is controlled by a "governor" of some sort which prevents the rate of catabolism from exceeding that of anabolism until the temperature reaches a certain value, at which point the cell dies

Earlier it has been pointed out that spores of the thermophilic bacilli are considered to be the most heat tolerant of all bacterial spores The body of data which has led to this conclusion is large, but the diverse and poorly controlled conditions under which most workers studied the thermal resistance of the spores of thermophiles, provides little basis for a comparison with the resistance exhibited by other species (e g, 23, 169) Eckford (71) and others (22) have pointed out a direct relationship between the maximum temperature for growth of a thermophilic organism and the heat resistance of its spores An analysis of a more recent controlled experiment (153) has indicated an inexact, but significant, correspondence of maximum growth temperature and thermal resistance of spores Other factors than those which determine maximum temperature for growth of the organism are believed to be involved in the phenomenon of thermal resistance

In accord with the above correlation, Eckford (71), Bergey (19), and Esty and Williams (78) have noted that spores of true thermophiles have a greater heat tolerance than spores of thermotolerant *organisms Black and Tanner* (22), however, have found that the spores of most thermophiles are not unusually resistant to heat and that a particular strain exhibited the same resistance to heat, whether the organism was *isolated directly* from nature or "selected" for heat resistance by isolation from sources previously heated or processed The spores of only two strains, of the many aerobic thermophiles studied, were found to survive 100 C for 24 hours, 115 C for one hour, and 120 C for 25 minutes Other investigators have noted cases in which spores of thermophiles have survived autoclaving (184)

NATURE OF GROWTH OF THERMOPHILES

1 Growth at Elevated Temperatures Growth of thermophilic microörganisms has been determined largely by visual observation Tanner and Wallace (251)

were the first to apply the quantitative growth-curve method to the thermophilic bacteria They prepared growth curves for three bacilli at 20, 37, and 55 C The lag phase at 55 C could be decreased greatly by pre-heating the medium and using an inoculum of young cells At 55 C, they observed most rapid increase in cell numbers and, after the period of active growth, a rapid death, and since cultures often became sterile, it may be inferred that these thermophiles did not sporulate at this temperature The absence of spores may have been the result of the very low oxygen tension in a liquid medium at an elevated temperature, in accord with the observed relationship of oxygen tension and the capacity to sporulate among the aerobes, as well as facultative and strict anaerobes (42)

Hansen (106) prepared growth curves of a strain of Cameron and Esty's facultative thermophiles Group 80 with the object of obtaining information about the generation time and rate of fermentation The growth rate was found to increase with increasing temperature to about 55 C, above which the rate decreased At the point of maximum viable cell number, the 55 C curve fell off much more sharply than the corresponding curve at 37 C A generation time of 16 minutes was reported at 55 C In the presence of glucose, and calcium carbonate to neutralize the resulting acid, the maximum viable cell yield (*ca* 6×10^8 per ml) was obtained at 42 C, rather than at 55 C, in addition, the crop decreased at temperatures above and below 42 C, but again became large at 20 C The viable crop represented only a fraction of the total count Hansen maintains that thermophilic cultures become sterile when stored at high temperatures if the acids formed from carbon compounds in the medium are not neutralized Although the yield of viable cells is low at 55 C (*ca* 10^8), the rate of fermentation is high enough to effect great chemical changes in a short time Hansen estimates that the fermentative capacity of this thermophile at 55 C is about thirty times as great as that of *Streptococcus lactis* at 20 C

Imsenecki and Solnzeva (129) and Gaughran (89) have been unable to demonstrate a lag with a number of thermophilic bacilli, when using inocula consisting of cells from 12- and 17-hour cultures, apparently in the maximum stationary phase of the cultural cycle In accord with the data of previous workers, growth was characterized by high reproduction and death rates The logarithmic growth phase, during which the rate of multiplication remains constant, is probably of very short duration and is not evident in the population curves The total population curve and the viable population curve rapidly diverge to a point at which the total count is 50 to 100 per cent larger than the viable count Such a divergence is indicative of a rapid death rate A pronounced negative slope of the latter portion of the total population curve has invariably been noticed It is first apparent at approximately the same time at which the number of viable cells begins to decrease Such a decrease in the total number of cells has been attributed to a cytolysis induced by autolysis or an accumulation of toxic products in the medium

The above observations of cytolysis, augmented by the occurrence of many

"ghost" forms, not included in the total microscopic count, leads to an entirely different interpretation of the population cycle Additional evidence of this autolysis has been found by Imsenecki and Solnzeva (129) in the very rapid accumulation of enzymes in the medium containing thermophilic organisms Thus, the generation time of the thermophilic bacilli is very short, perhaps of the order of magnitude of 5 to 15 minutes, and not several hours as indicated by the curves determined in the usual way The curves obtained represent only a quantitative expression of the numbers of living and dead cells present at any one time in the culture, and gives no indication of the rate of reproduction Autolysis is present early in the culture cycle and becomes a predominant factor in the latter portion of the population curve Thus, autolysis effects a lowering of the total population curve and depresses the apparent death rate

Cultures of stenothermophiles, as a result of a high death rate and autolytic rate, never reach the maximum viable or total populations so common in most mesophilic cultures In the aerobic cultures the oxygen demand of the rapidly metabolizing cells can not be supplied adequately even in a very shallow layer of medium The ability of aeration to increase viable cell yield seems to bear some relation to the temperature range for growth of the organism, for thermophiles with a broad temperature range for growth respond to a greater degree than thermophiles with a narrow temperature range

Imsenecki (123) has found that proteolysis, denitrification, and hydrolysis of starch by thermophiles proceeds at a rate seven to fourteen times that of cultures of mesophilic bacteria A study of culture populations and the rate of biochemical activity indicate that the high reproductive rate is inadequate in explaining the intense biochemical activities of the thermophilic bacteria In the case of proteolytic thermophiles in suitable media, the number of viable cells rapidly increases (to *ca* 80×10^6) and then decreases according to the typical population curve discussed above, while the proteolytic activity gradually increases and reaches a maximum at a time corresponding to the lowest portion of the viable population curve (*ca* 10×10^6) A mesophile, *Bacillus mesentericus*, on the other hand, shows the usual increase in protein digestion with increase in the number of viable cells The progress of proteolysis may, of course, be related to the rapid reproduction, death, and autolysis, and a resultant accumulation of proteolytic enzymes in the medium The behavior of amylolytic thermophiles, however, according to Imsenecki (123), does not present a corroborative picture Here diastatic activity proceeds at a rate far out of proportion to the number of viable cells in the culture and increases very rapidly during that period in which autolysis is not a predominant factor in the culture cycle Thus Imsenecki is led to the conclusion that an explanation of the high biochemical activity of thermophilic bacteria is to be found in the very intense metabolic activity of these organisms, and not merely in their rapid proliferation Russian workers have conducted a number of investigations of the amylolytic (123–5, 128, 130), cellulolytic (121, 122, 127, 229–231), and proteolytic (123) thermophilic bacteria with an appreciation of the potential value of these organisms in industrial application

The unfavorable effects of the high temperature at which thermophilic bacteria have their designated optimum have frequently been pointed out Suggestions have been made that, perhaps, the optimum temperature of these so-called thermophiles should be considered as much lower A number of thermophilic strains have been found to die out if continuously cultivated or even stored at elevated temperatures The inability to form spores has usually been held responsible in the case of facultative thermophiles Thermophiles with a narrow temperature range, however, do produce spores at their optimum, but experience a depression in this activity as the maximum temperature is reached Thus, it is possible that rapidly metabolizing vegetative cells in a depleted medium and a high concentration of a toxic metabolic product, such as acid, would be unable to survive for long, if the cells were unable to produce some resistant form Experience has shown, however, that in the absence of detrimental metabolic products, thermophilic cultures can be stored indefinitely at temperatures of 50 to 60 C

Considerably less uniformity in size and proportion has been noted in thermophilic cells cultivated at their optimum temperature than when cultivated at lower temperatures (261, 110, 208) At 55 C many of the thermophilic cells become long, slender, frequently curved, and show marked granulation, at 37 C, cells of the strains examined are smaller, more uniform in size, and stain homogeneously Pleomorphic forms in cultures of thermophilic microörganisms have been described by various observers (23, 135, 234, 236) The microscopic appearance of the organisms cultivated at different temperatures, thus, has led many workers to believe that the real optimum temperature of the thermophilic forms is much lower than generally assumed

The selection of the optimum temperature for a thermophilic form, of course, depends upon the criterion selected Whether we select the region of maximum viable cell yield or maximum reproductive rate is unimportant, provided the latter is compatible with the preservation of the species For any organism there will be a discrepancy between the temperature at which these two maxima occur, and it is apparent that this discrepancy will vary directly with the breadth of the over-all temperature range for growth It has been the recent practice to use the point or range of highest reproductive rate in the designation of the optimum temperature for growth of thermophiles

2 Growth at Low Temperatures The thermophilic microörganisms, arbitrarily characterized by an optimum temperature above 50 C, exhibit considerable latitude in their over-all temperature range for growth A large number of thermophilic bacteria have been found to grow at 37 C, and even at 20 C An equally great number of cultures have a very high minimum temperature for growth The fact, that the latter organisms were found in geographic regions where their minimum temperature for growth was seldom, if ever, reached, gave rise to a lengthy and heated argument with regard to the growth characteristics of the thermophilic microörganisms

One school maintains that all organisms which grow at high temperatures are thermotolerant, i e , thermophiles grow at high temperatures and also, more

slowly, at lower temperatures Here adaptation to an elevated temperature range is never complete, and, many cases (2, 144, 251, 106) have been described to substantiate this belief Evidence (234, 193, 182, 144) also has been presented to show that the environment (culture medium) exerts an important influence on the temperature limits for growth, and may be responsible for the inability of many workers to obtain growth at both ends of the temperature range Although scattered evidence of this effect on thermophilic bacilli occurs in the literature of the last century, the first controlled experiments were conducted in 1911 by Koch and Hoffmann (144) They found that thermophilic bacilli isolated from the soil would not grow in artificial culture media at temperatures below 40 C, but grew well in soil at temperatures as low as 20 to 30 C Thus, they derided Fischer's theory of dormancy of thermophiles (82) and Miehe's explanation that thermophiles in temperate regions required heated piles of organic matter for growth (171) Thermophilic bacilli, according to Koch and Hoffmann, proliferate at low temperatures when in their native environment Noack (188) concedes such a possibility in the case of bacteria, but not of molds The influence of the composition of an artificial culture medium on the temperature characteristics of thermophilic bacteria has been studied by other workers (182)

Rabinowitsch (215, 216) found that thermophilic bacteria that she isolated grew at 33 C if cultured anaerobically, whereas no growth was evident aerobically at this temperature Thus she was led to conclude that oxygen tension was the factor which determined the minimum temperature Her findings were confirmed, in part by Schütze (238) and Ambrož (2), but opposed by the results of Oprescu (193), Miehe (171), de Kruyff (150), and Shaw (243) Nègre has concluded from his studies that all obligate thermophiles are obligate aerobes and all facultative thermophiles are facultative aerobes Recent studies (42) have revealed that there is considerable diversity with respect to the effects of temperature upon oxidation-reduction relations, which would indicate that the results of Rabinowitsch are limited in scope Since the time of Rabinowitsch, thermophilic species have been found with oxidation-reduction potential requirements representative of aerobes, facultative anaerobes, and anaerobes (80, 38, 165, 166)

Morrison and Tanner (181, 182) have suggested that in the observation of growth of thermophilic bacteria at low temperatures, the time element is of greatest significance They maintain that many investigators have not waited long enough for proliferation to become apparent, and have concluded incorrectly that the organisms were incapable of growth. Quantitative studies (106, 251) indicate that thermophilic bacteria multiply so slowly at low temperatures that an increase in number of cells has been overlooked consistently Hansen (106) found a generation time of 370 minutes at 20 C for the thermophile he studied Therefore, when dealing with thermophilic organisms, the ordinary method by which an inoculum is spread over an agar slant and observed for growth after a period of incubation, was considered inadequate Others (89, 129) have proved that the apparent failure of proliferation in some cases is not the result of the insensitivity of the method

It is apparent that this school had little difficulty in fitting their thermophiles into the economy of nature in temperate zones, and repeated emphasis has been placed upon the importance of their activity in the soil (3), surface waters (8), and the intestine (215)

The second school recognized two groups of thermophiles one with a wide temperature range, including the usual mesophilic range, the other with a narrow range, the lower limit of which is usually above 30 C These groups may be designated by the terms "eurithermal" and "stenothermal", respectively

Although it was not until the past decade that a relatively clear picture of the latter group was presented (129), the literature contains numerous accounts which satisfy the definition of the stenothermal type But we find also that we cannot select an arbitrary temperature as the minimum temperature for growth of all stenothermal organisms and thus differentiate these two types of thermophiles An undeniable transition between the eurithermal and the stenothermal groups is indicated

Many of the twenty species of MacFadyen and Blaxall (163) had minima between 60 and 65 C, Bergey's (19) *Bacillus thermodiastacus* and *Bacillus thermononliquefaciens*, 50 C, *Bacillus thermophilus vranjensis* of Georgevitch (93, 95), 49 C A minimum of 45 C was found for the organisms of Hussong and Hammer (119), Donk (66), Gilbert (96), and Georgevitch (94), 42 C for *Bacillus pepo* of Shaw (243), and, 40 C for the species of Sames (234)

Many other organisms with a minimum temperature between 37 and 45 C have been described (150, 44, 258–260, 170, 79)

Bergey (19) has described organisms, such as *Bacillus thermoliquefaciens*, *Bacillus lobatus*, and *Bacillus thermotranslucens*, which show slight growth at 37 C *Bacillus thermocellulolyticus* of Coolhaas (53) was found to have a minimum of 35–37 C Schütze (238), Miehe (171), and Kedzior (139) have described thermophiles with temperature minima in the region of 30 C

However, the clarity of this gradual transition does not remove the question of the existence of thermophiles with very elevated minimum temperatures The suggestion that failure of proliferation below a critical temperature is more apparent than real, has been cited earlier The time element in incubation of thermophiles at temperatures below their apparent minimum has been shown to be unimportant in the case of several typical strains of obligate (stenothermal) thermophiles studied by Cameron and Esty (38) Dextrose broth and corn juice inoculated with spores of thermophilic bacilli, with a minimum temperature of 42 to 45 C, showed no activity during five years of incubation at 35 to 37 C Similar inoculations into canned corn, held at 22 C and 37 C, exhibited no activity during three years of incubation All samples, however, when placed at 55 C invariably gave rise to rapid proliferation and acid production Analogous results were obtained by Shaw (243), using *Bacillus pepo*, in studies of shorter duration Recent work by Curran and Evans (56) indicates the necessity for preliminary heat-shocking of spores of thermophilic aerobes before germination will proceed Additional observations of the rapid loss in vitality of heat-activated spores, when subjected to an unfavorable environment, (57) provides a possible explanation of the frequent notation that thermophilic cultures become sterile when stored at room temperature

The early literature on the survival of thermophilic microörganisms at subminimal temperatures contains many inconsistent statements Thus, Tsiklinsky (359) found that her spore-free cultures of *Thermoactinomyces vulgaris* survived storage at a subminimal temperature, while Miehe (171) observed the rapid death of spores of *Actinomyces thermophilus* in artificial culture media Similar opposing results have been presented for spore-free cultures of thermophilic bacilli at temperatures below their apparent minimum (171, 234) Noack (188) investigated the effect of subminimal temperature on the vegetative cells and spores of five thermophilic molds, a thermophilic actinomycete, and a thermophilic bacillus The vegetative forms of the three molds and the bacterial species exhibited very low resistance to a temperature slightly below the minimum, while the other two mold species and the actinomycete showed a very low death rate under the same conditions Spores of all forms, however, were highly resistant The susceptibility to the effects of low temperature bore no relation to the minimum temperature for growth of the organism in question, to the temperature of incubation prior to storage at a subminimal temperature, or to the composition of the culture medium Death at such temperatures was explained by the assumption of a unique respiratory system, as suggested earlier by Miehe (171), or a membrane with a very critical response to decrease in temperature

Shaw (243) has another explanation of the observed effect of low temperature on thermophilic bacteria Her cultures, when stored for a prolonged time at room temperature failed to yield viable inocula Subsequent study revealed that upon storage, turbidity disappeared from tubed liquid media and samples taken from the upper portion of the media proved to be sterile in many cases, while samples removed from the bottom portion yielded viable spores The tendency of the culture to sporulate and of the spores to settle out permitted a number of conclusions

The occurrence of bacteriophage active against thermophilic bacilli (1, 145) has suggested phage as possible growth inhibitor, effective at lower temperatures Abundance of growth at elevated temperatures could be explained by the heat-lability of the phage in question Partial inactivation of the phage at the minimum temperature for growth of the organism would account for the observed effect However, phage with these unusual properties has not been detected in cultures of thermophilic bacteria

Quantitative population studies undertaken by Imsenecki and Solnzeva (129) have established that workers, who have denied the existence of bacteria of the stenothermal type and have claimed that all thermophiles proliferate at low temperatures, have not had the opportunity of examining members of the stenothermal group Gaughran (89), in a study of five stenothermophilic bacteria has demonstrated that the environmental factors, such as, nutrient and nutrilite supply, inhibitors, oxygen and carbon dioxide supply, oxidation-reduction potential of the medium, relative hydration and pH of the medium, exert a considerable influence on the growth response of these organisms within the temperature range of approximately 38 to 75 C Proliferation did not occur

under any combination of conditions at temperatures below 38 C Manometric data, used as a measure of growth, in all cases paralleled population data The behavior of the stenothermophilic bacteria suggests peculiarity in their enzyme complement or, perhaps, a unique structural chemistry

Analyses of the minimum temperature for growth have been predicated more on hypothesis than on experimental data Ideal material for the study of this fundamental problem is found in the inability of the stenothermal thermophiles to metabolize and reproduce at temperatures which are suitable for most other forms of life Generally, attempts at an explanation have not gone beyond the suggestion that these organisms possess a unique mechanism, which has apparently replaced the mesophilic mechanism lost during the adaptation process One study (71) has indicated that the respiratory enzymes of thermophilic forms do not function at ordinary temperatures, others (111, 152) suggest deficiencies in the respiratory and hydrolytic systems

Growth processes and processes furnishing energy are usually placed in separate categories and their interdependence frequently minimized Although both processes are conceded to be enzyme-catalyzed, the temperature range of the energy-yielding processes is much wider than that of growth processes Reactions involving energy liberation and syntheses are so interlinked that the retardation of any single reaction might prevent completely the functioning of others and thus make growth impossible The absence of growth and proliferation in stenothermophilic cultures may thus be related to the failure of one or more steps in this metastable chain of exothermal and synthetic reactions Synthetic reactions in biological systems are still obscure and our knowledge is based largely upon the isolated endproducts and a few intermediate compounds The respiratory mechanism, including all chemical processes by which energy is made available to the cell, has been studied extensively in some animals and plants Investigation of the activity of the bacterial respiratory enzymes with respect to temperature has been confined largely to organisms with relatively low minimum growth temperatures References made by several workers to the inactivation of bacterial enzymes by low temperatures and to deficiencies, both qualitative and quantitative, in the respiratory mechanism of thermophiles have no supporting experimental data

Foter and Rahn (85) in an analysis of minimum temperature state that the most common explanation of cessation of growth at low temperatures is the assumption that the numerous interlinked reactions of the cell are influenced differently by a change in temperature, with the result that the growth mechanism is upset The accumulation of toxic metabolic products within the cell is not considered a possible cause for a disturbance of the growth mechanism in the case of bacteria or other cells with large surface area Excessive viscosity of the protoplasm is also discounted The change in permeability induced by changes in temperature is cited as a possible explanation and its relation to the consistency of lipids suggested In an accompanying laboratory study they demonstrated that lactose fermentation by several lactic acid organisms takes place at a temperature below the apparent minimum temperature for growth

A more recent study of the stenothermal *Bacillus cellulosae-dissolvens* (129) indicates that this situation is not encountered invariably Flasks containing cellulose were inoculated with the organism and incubated at a high temperature until fermentation was well advanced Then a sample was removed, and the flasks placed at 20 C for ten days No change in quantity of the hydrolytic products of cellulose or volatile acids could be detected However, all the extracellular hydrolytic enzyme preparations from thermophilic bacteria which have been examined to date exhibit activity at ordinary temperatures (91)

In a recent kinetic study of the effect of temperature on the respiratory mechanism of the stenothermophilic bacteria (91), the respiratory mechanism and its various enzymic components were found to function at temperatures far below the minimum for growth In every case the rates of the individual reactions involved in the respiratory chain increased exponentially with temperature up to the temperature at which inactivation became apparent Identical energies of activation for the over-all respiratory system and its enzymic components were obtained at temperatures above and below the minimum temperature for growth of the organisms This observation is significant in the indication that there is no fundamental difference in the effect of temperature on the respiratory systems of stenothermophilic and mesophilic bacteria The similarity in nature of the enzymes functioning in the respiration of mesophiles and thermophiles also is suggested

The stenothermal thermophiles have been used to test the general hypothesis of Heilbrunn (112) and Bělehrádek (15) which related heat resistance of an organism and the melting point or degree of saturation of its protoplasmic lipids (90) The results of this study suggest a possible extension of this hypothesis, namely, that the temperature range for growth is a function of the degree of saturation of the cellular lipids A large proportion of the cellular lipids of the stenothermophilic bacilli was found to exhibit a high degree of saturation over the entire temperature range for growth Thus, as the minimum temperature for growth is reached a large proportion of the lipids approach solidity The incompatibility of this situation with active metabolism at lower temperatures has been pointed out, as well as the inference that the consistency of the fats elaborated by the stenothermophilic group of bacilli may prevent active metabolism at low temperatures and fix the minimum temperature for growth

TEMPERATURE ADAPTATION OF MICROÖRGANISMS

The assumption that thermophilic forms were the result of an adaptation process stimulated short-term adaptation experiments employing both plants and animals On the suggestion of Darwin, Dallinger (60) studied three flagellates and cited the results in his presidential address before the Royal Microscopical Society in 1887 These protozoa, which originally grew at about 16 C and had their maximum at 23 C, by gradual exposure to increasing temperature over a period of seven years, were made to grow normally at 70 C Davenport and Castle (62), by incubating frog eggs at temperatures of 10 C above normal, obtained tadpoles with a temperature tolerance 3° above normal

Signs of adaptive changes have also been found in Fundulus, insects, coelenterates, protozoa, human erythrocytes, and isolated frog nerve (16).

Dieudonné (64, 65) observed an elevation in the growth temperature of *Pseudomonas fluorescens* and of *Bacillus anthracis* during his study of the behavior of various bacteria to unfavorably high temperatures. Similar response, although very slight, has been reported for other bacteria (260, 233, 203, 183, 167) and molds (256). Attempts at an adaptation, over a period of a year, of ten spore-forming mesophilic bacteria by Casman and Rettger (42) were unsuccessful. Desiccation, and growth in concentrated solutions of sucrose, peptone, or sodium chloride also failed to increase heat tolerance (42, 72). "Temperature shocking", a selective process, has been successful in shifting the maximum growth temperature of two non-sporeforming bacteria several degrees (32).

Jancke (135), noting a similarity between the thermophilic organisms and the *Bacillus mesentericus* group (fuscus, ruber, vulgatus, panis viscosi), attempted to develop heat resistant strains from the mesophilic species, as well as to adapt heat resistant strains to lower temperatures. Unfortunately, all the "thermophiles" which he developed, and all except one which he isolated, would not survive cultivation at 60 C for more than two or four transfers. In addition, at this temperature they lost their ability to produce spores and coagulate and peptonize milk. They were indeed similar in character to species of the "mesentericus group" cultivated at 55 to 60 C, but they failed to fulfill the author's definition of a "thermophile". One organism which he isolated and designated as an obligate thermophile grew optimally at 60 C for many transfers and never yielded a strain which would grow below 40 C. Jancke was attempting to provide data for the theory of Lieske (159) which maintained that a radical or sudden change in temperature was capable of spontaneously inciting a mutation, thus adapting the organism to the new environment. Both Jancke and Lieske, however, admit that the spontaneity of this "mutation" may be more apparent than real.

An appreciable increase in temperature tolerance appears to require a great length of time, and the sudden development of thermophilic forms as suggested by Lieske (159) and Kluyver and Baars (143), frequently has been considered as very doubtful (129). Kluyver and Baars offer the interesting implication that many thermophilic cultures are physiological artefacts. This is based upon a study of *Vibrio thermodesulfuricans*, supposedly derived from *Vibrio desulfuricans*. The thermophilic organism is strictly anaerobic and does not form spores. Minute amounts of oxygen sterilize the culture as the temperature of cultivation approaches the minimum and the rate of metabolism falls off. The organism is considered to be a physiological artefact, because it is obviously unsuited to occur in nature.

Starkey (248) has stressed the significance of the results of Kluyver and his students from their studies of the effect of environment on the characteristics of microörganisms. The effect of temperature on the morphology of the vibrios and spirals, and the ease with which adaptation occurs in these forms cannot

be minimized Further clarification of these observations was found in the course of an investigation of a sporogenous vibrio, *Sporovibrio desulfuricans* Beijerinck (247, 248) The very wide temperature range for growth of the vibrios and spirals (*ca* 40 C) suggests that other bacteria can be made to grow at temperatures well above the generally accepted maximum temperature by a gradual adaptation process Thus, by gradually altering the temperature of incubation, the cardinal temperature characteristics for growth (i e , minimum, optimum, and maximum temperatures) of a particular organism can be changed Such a general situation would detract from the significance of these terms

The "lipoid liberation theory" of Bělehrádek (15), which links the heat "adaptation" of the protoplasmic fats with the adaptability of the whole organism to high temperatures, has been substantiated in part by the work of Fraenkel and Hopf (86) The results of their work led to the suggestion that although the physical nature of the lipids may have a decided influence on the chain of physiological processes, the theory is entirely inadequate in explaining the phenomena of heat injury and heat adaptation

Explanations of thermobiosis based on the assumption that thermophilic organisms possess a unique mechanism, which apparently replaces the mesophilic mechanism lost during the adaptation process, has led to a further assumption of enzyme adaptation Although the question of temperature adaptation of biological "ferments" was under consideration (81) long before the term "enzyme" had been proposed, sight of the basic problem has been lost in later years Some workers (142) have been led to believe that entirely different enzymes are concerned in the metabolism of cold- and warm-blooded forms, although it has frequently been reported (148) that the enzymes of cold-blooded animals at lower temperatures are as active as the enzymes of warm-blooded animals at higher temperatures The classical example of a typical enzyme adaptation, presented by Kjeldahl (141) in 1881 for invertase of top and bottom yeast, was accepted for thirty years (192), until disproved by von Euler and his students (272, 273) Harder (109) has claimed indirect evidence for the adaptation of assimilatory enzymes of one of the higher plants to the temperature of the environment, one of his students (197, 162), however, upon extending this work to the diastase of *Aspergillus niger* and *Penicillium glaucum* in a poorly controlled experiment, concluded that there was no adaptation of enzymes to change in temperature at which these enzymes were formed

When we realize that plant enzymes in general exert their optimum activity between 50 and 60 C (260), although they are seldom subjected to such temperatures in nature, the optimum growth of bacteria at 50 and 60 C, and continued growth at even higher temperatures is understandable to some degree However, among the thermophiles it is true that the few enzymes which have been studied in growing cultures and resting cell preparations apparently have slightly higher optimum and maximum temperatures than mesophilic forms (91, 130, 284) A study of the activity of an extracellular hydrolytic enzyme in a growing culture, where the replacement of inactive enzyme molecules is an important factor, cannot reveal, however, the temperature characteristics

of the enzymes in question. Although there is no conclusive evidence that the enzymes of thermophiles have higher optimum and maximum temperatures than corresponding enzymes of mesophiles, the similarity of response of the respiratory enzymes of these two groups of organisms to change in temperature has been demonstrated (89). There is also no direct evidence that the enzymes of these organisms, or of any other organisms, have arisen as the result of adaptation of enzymes to temperature.

Attempts at lowering the cardinal temperature characteristics of an organism have been made, but these studies provide very little dependable data. Repeated freezing and thawing was ineffective in lowering the minimum temperature for growth of the cholera and typhoid bacteria (25). Similar failures, by gradual decrease in the incubation temperature over a long period of time, also have been reported in efforts to depress the optimum temperature of thermophilic bacilli (99), and the minimum temperature of *Bacillus anthracis* (64), of a thermophilic actinomycete (96), and of a thermophilic mold (188). Loss of the ability to grow at high temperatures upon prolonged cultivation in their lower temperature range (161), as well as the loss and subsequent recovery of thermal resistance (70), has been reported for a number of thermophilic microorgansims. A small number of such observations have been taken as evidence for the instability of the thermophilic species, and the reversibility of the adaptation of thermophiles to temperature is assumed commonly (99).

That some adaptations to environmental conditions occur fortuitously by mutation cannot be denied. The probability, however, of observing such a change by prolonged cultivation of an organism under a slightly modified environmental condition is hardly to be expected. Attempts at accommodation by gradual change of temperature in an effort to duplicate the process of natural selection are also unjustified, as attested by the very questionable success in the work undertaken in the past. The behavior of the vibrios in their ease of adaptation to changes in temperature must be considered unique, on the basis of our present knowledge.

SUMMARY: IMPORTANCE OF THERMOPHILIC MICROORGANISMS

The preceding examination of our accumulated knowledge of the thermophilic microörganisms suggests that the paradox in their behavior is more apparent than real. The similarity in the physiology of mesophilic and thermophilic bacteria is evident. Differences are to be found only in the intensity with which biochemical changes are effected. The gradual and imperceptible transition from the thermophile to the mesophile also suggests that the thermophiles do not represent an isolated biological group. As such, they constitute very significant material for study in order to augment our present very meager knowledge of the mechanisms involved in thermobiosis. Organisms of the group which exhibit an elevated minimum temperature for growth offer excellent material for clarifying the temperature responses of the growth processes. Although the behavior of this type of thermophile in pure culture is incompletely explained, a study of the activities of these organisms in nature may provide an

important contribution to our knowledge of symbiotic relationships among microörganisms It has been the aim of this review to present a complete picture of the scattered knowledge of the thermophiles against the background of a vast number of problems which they present, as well as to emphasize the importance of these organisms for study

Today thermophilic microörganisms come to our attention largely because they are responsible on occasion for the spoilage of processed foods Although many thermophiles were isolated from canned food early in the history of the canning industry, attention to these organisms as the cause of spoilage began with the work of Barlow in 1912 (9) Since that time the number of descriptions of isolation of the organisms and the types of spoilage has become so great as to make even a tabulation prohibitive The subject has been discussed and reviewed on many occasions (165, 249) Particular attention has been given to the presence of thermophilic bacteria in the various ingredients, such as starch and sugar, entering into the manufacture of foods (35–37, 43, 50, 249) The thermophilic bacteria were of great importance during the war in the case of certain canned foods designated as "commercially sterile", which rapidly spoiled upon storage in the tropics (10)

Rather recent recognition was made of thermophilic bacteria in milk, as a result of the sporadic appearance of large numbers of so-called pin-point colonies in the plate counts of pasteurized milk Since these organisms proliferate during the pasteurization process, a sample of milk may have a higher bacterial content after pasteurization The significance of these bacteria lies in their ability to ferment lactose, or less commonly decompose proteins, and cause undesirable flavors or odors Thermophiles are responsible to a large extent for pin-point colonies, although it has been shown (209) that other types of bacteria are also involved Many health authorities are inclined to disregard heat resistant organisms in milk because they are non-pathogenic, others maintain that the number of thermophilic and heat resistant bacteria constitute a good index of undesirable conditions in the production of milk and that the organisms can be controlled by the observance of rules of cleanliness Since the standard plate count does not reveal all thermophilic bacteria present in a sample, and therefore does not present an accurate picture of the bacterial condition of the milk, other routine methods of examination have been proposed (249)

In addition to the undesirable activities of the thermophiles, the beneficial aspects of these organisms also must be noted They have been considered earlier in the discussion as potential agents in the controlled fermentation of cellulose to useful products The versatility of the intense biochemical activity of thermophilic microörganisms, taken as a group, offers many opportunities for their industrial application Thermophiles have found application in the recovery of vegetable oils and fats (12) and in the degumming of silk (138) The high metabolic rate of thermophiles which results in rapid accumulation of a large quantity of extracellular enzymes in the medium is also a situation rife with possibilities Amylase (125) and "degummase" (128) of thermophilic bacilli have found industrial use as enzyme preparations

It is obvious that the problems of thermobiosis are not purely academic, nor are they of interest to industry merely because of the presence of thermophilic bacteria in foods and products. Thermobiosis may perhaps also provide a tool with which desired biochemical changes may be effected more rapidly.

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A PUBLICATION OF THE
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EDITOR
BARNETT COHEN

*It is characteristic of Science and Progress
that they continually open new fields to our vision.*—PASTEUR

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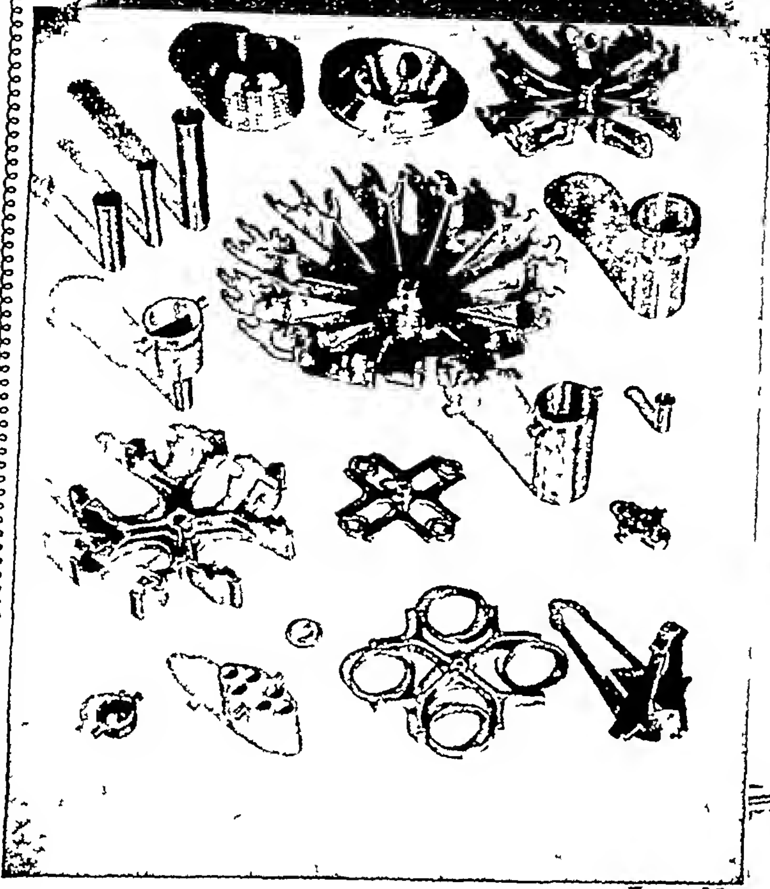

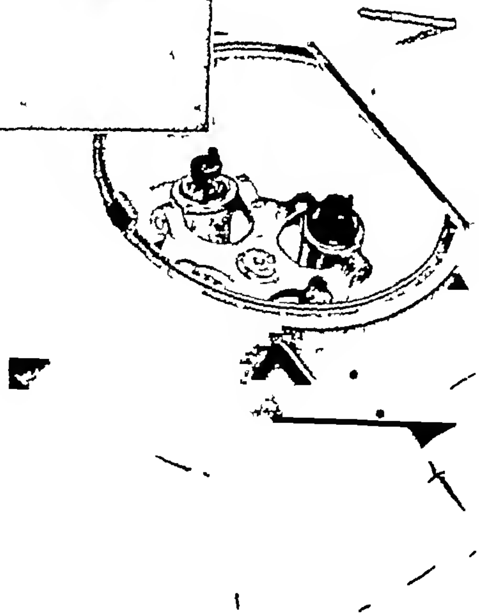

THE YEAST-LIKE FUNGI: CANDIDA AND BRETTANOMYCES

C. E. SKINNER

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In 1941 Henrici (111) published in this Journal an extensive review on the genetics, cytology, variation, classification and identification of yeasts. That review was "built up around" two monographs "Die sporogenen Hefen" by Stelling-Dekker (261) and "Die anaskosporogenen Hefen. Erste Hälfte" by Lodder (159). The third of the series "Die anaskosporogenen Hefen. Zweite Hälfte" (69) by Diddens and Lodder was distributed in the United States late in 1945, although it bears the publication date 1942. This volume deals with yeasts lacking a sexual ascogenous stage and forming pseudomycelium and often also true mycelium. The present review will be centered, to some extent, around this last monograph, as well as around the "Zimología Médica" of Mackinnon (168) published in 1946, which deals more especially with the species of medical interest. The emphasis will be on the literature published since 1930. Due to lack of space, several papers dealing with the medical aspects of the genus *Candida* have had to be omitted from consideration.

TAXONOMIC POSITION OF YEAST-LIKE FUNGI

The "yeast-like fungi" have perhaps given as much trouble to microbiologists as any group of microörganisms. Since they are studied by a technique essentially bacteriological, mycologists have frequently left them to bacteriologists for study, who in turn, since these organisms actually are Eumycetes, often tend to refer them back to mycologists. We can give a precise botanical definition to the organisms in question even less accurately than Henrici could define a yeast. They are a group of microörganisms probably of mixed phylogeny and of varied morphology and they merge into various genera of perfect and imperfect fungi. They show relationships to Saccharomyces, Pichia, Endomycopsis, Endomyces and possibly other perfect yeasts, to Cryptococcus (Torulopsis), Mycoderma, Rhodotorula and other imperfect yeasts, and according to Diddens and Lodder to Sporotrichum, Geotrichum (Oöspora) and other imperfect molds. We are justified in calling them "yeast-like" since they produce yeast-like daughter cells by budding from the mycelium. Because of the tendency of these daughter cells

to continue to produce more yeast-like cells by the same process, the yeast cells often predominate in cultures. Yeast cells produced by such budding are known as blastospores. Some species also produce, by a process of disarticulation of the mycelium, more or less yeast-like daughter cells known as arthrospores or oidia.

In spite of their wide distribution, great economic importance and interesting diversity of morphology and physiology, the yeast-like fungi have remained, in general, poorly understood and partly as a result of this, and partly as a cause of it, they have been poorly and inadequately classified. The Dutch monographists have done a great service to science in preparing the three volumes mentioned. Their contribution, although almost exclusively taxonomic, becomes even more apparent when it is realized that they studied thoroughly, both morphologically and culturally, hundreds of separate cultures from all over the world. A great many of the cultures were types. Diddens and Lodder's work then is not only a critical study of the literature, but it represents also an enormous amount of laboratory work extending over many years on the part of their institutions, the Centraalbureau voor Schimmelcultures, Baarn, and the Technische Hoogeschool, Delft, Netherlands.

We may recognize three families of imperfect yeasts. In the family Sporobolomycetaceae the multiplication is by budding and also by "*repetition*", a process in which cells forcibly propel a daughter cell from a *sterigma*, like the basidiospores of the Basidiomycetes are propelled. Actually, Stelling-Dekker placed the Sporobolomycetaceae in the class Basidiomycetes. To classify them thus, the forcibly propelled cell must be considered a sexual spore having originated from a union of nuclei with subsequent reduction division. The writer knows of no adequate proof of such sexuality and so, following G. W. Martin (180), considers the Sporobolomycetaceae as probably imperfect stages of certain Basidiomycetes, hence Fungi Imperfecti. The Sporobolomycetaceae may or may not produce a pseudomycelium and they never ferment sugars. The second family, the Rhodotorulaceae are like the preceding in that they never ferment sugars and in that they do possess carotinoid pigments. There is very strong evidence that the Rhodotorulaceae have descended from the Sporobolomycetaceae, and that the process of repetition has been lost. The third family is the Cryptococcaceae, called the Torulopsidaceae by Diddens and Lodder. Since we do not recognize the genus *Torulopsis*, we do not use the family name Torulopsidaceae. Cryptococcaceae has long been used as a family name. The Cryptococcaceae are yeasts lacking a perfect stage, without carotinoid pigments, reproducing by budding, but not by repetition. Pseudomycelium, a structure produced by budding cells elongating and clinging together in chains, thus resembling true mycelium, may or may not be formed. Sugars may or may not be fermented. A fourth family, the Nectaromycetaceae has quite properly been relegated to the third by Diddens and Lodder.

The Cryptococcaceae are divided into two subfamilies, the Cryptococcoideae with no or only slight pseudomycelium and the Candidoideae with well developed pseudomycelium. These two subfamilies were called Torulopsidoideae and Mycotoruloideae by Diddens and Lodder, but these names are based on generic

names which we do not accept and have been corrected in accordance with the common interpretation of the International Rules of Botanical Nomenclature, the latter by Buchwald (26), the former by the author[1] (253)

Henrici (111) called attention to the fact that the primary division of yeasts which produce mycelium from those which do not is one that is difficult to make in practice Most yeasts, if they are studied sufficiently, will be found to produce pseudomycelium or sometime possibly even true mycelium There are all gradations between no pseudomycelium at all and a pseudomycelium so well developed that it can hardly be differentiated from true mycelium except by following through its formation as it develops in the microscopic field Stelling-Dekker avoided this difficulty by classifying together ascosporogenous yeasts with and without mycelium Lodder chose to divide the Cryptococcaceae (Torulopsidaceae) into two subfamilies on this basis Frankly, the author does not like this division but must confess that he cannot suggest a better one It is evident that this has given Diddens and Lodder some difficulty also

Diddens and Lodder's subdivision of the Candidoideae into only three genera is both logical and convenient It is made on the basis of the type of thallospores produced, blastospores or arthrospores Forms which produce blastospores only are put in the genus *Candida* or the genus *Brettanomyces*, those which produce both blastospores and arthrospores are put in the genus *Trichosporon*, those which produce arthrospores but not blastospores are excluded from the family These include such organisms as *Geotrichum candidum* (*Oidium lactis*)

OLD AND RECENT CLASSIFICATIONS OF CANDIDA

Candida albicans (Robin) Berkhout is not only the type species of the genus but it is the outstanding species in importance and interest The confusion in the nomenclature of this species may be appreciated from the fact that Ciferri, Redaelli and Cavallero (52) in 1938 found 45 different binomial synonyms, Diddens and Lodder in 1942 (69) cite 87, and Conant and associates, in 1944 (56) state that there are 172 in the literature!

It was just 100 years ago, in 1847, that Robin first described a species of *Candida* It is possible that Langenbeck and other earlier workers had seen the fungus, but their descriptions do not appear to be sufficiently accurate to show what organism was encountered Six years after Robin had published his thesis, he gave the organism its first Latin binomial, *Oidium albicans* Recognizing the fact that this organism did not belong to the genus *Oidium*, Quinquaud (230) renamed it *Syringospora Robinii*[2]

Various other generic names, especially *Monilia* have been used in the past

[1] *Cryptococcoideae*, nomen novum Nomine subfamiliae sensu "*Torulopsidoideae*" Diddens et Lodder utor

[2] International Rules of Botanical Nomenclature recommend that specific names derived from personal or generic names begin with a capital letter Bacteriologists rarely follow this recommendation but most mycologists do so Since the forms under consideration are definitely true fungi, not bacteria, the writer will use capital letters where indicated but such use does not necessarily reflect his personal preference

for the genus *Candida* as a whole, or for portions of it. The use of *Candida* as a generic name to replace the familiar, but always invalid *Monilia* can be justified neither by its legality nor by extensive past usage. *Syringospora* is the oldest valid name for the genus (55, 168). The original description and drawings of *Syringospora* (unusually good for 1868) are definite enough for one to be reasonably certain that the organism now known as *Candida albicans* was referred to. Quinquaud's misinterpretation of structure is of no taxonomic consequence. The only other generic name that need concern us here is *Monilia* under which name the most of the past literature will be found. For about a century the term *Monilia* has been used for many organisms but principally for two entirely different sorts of fungi: for the imperfect stage of certain ascomycetous plant pathogens and for certain yeast-like fungi such as the causative agent of thrush. At the present time practically all mycologists (Dodge, 70, is an exception) agree that the former usage is the correct one and have substituted other generic names for the other group. At an informal meeting of those interested in the subject, at the Third International Microbiological Congress in 1939, delegates agreed to use *Candida*, although it will probably be necessary to have the name validated as a *nomen conservandum* by a Botanical Congress. Most workers have accepted this decision, and all bacteriologists and medical men, we believe, will do well to do the same in the interests of uniformity.

Unfortunately, there are a score or so of other generic names in the literature that have been used for the whole group or parts of it. Ciferri and Redaelli (49) proposed a classification which recognized six genera of organisms that produced blastospores only, one genus with both blastospores and arthrospores, and one with arthrospores only. Later, another classification by Langeron and Talice (152) divided the blastospore producing species into six somewhat differently conceived genera. Although these classifications were finally discarded by Diddens and Lodder (67, 69)—earlier, Lodder (159) had expected to follow them—much of our present day precise knowledge of the morphology of this group is due to the work initiated by these men.

It was the almost classical paper of Benham (18) which indicated the basis on which the "monilias" were to be classified. Benham's original work and critical survey of the literature showed that neither a purely morphological nor a purely biochemical classification was satisfactory. By selecting those characters which were important, combining them, and correlating results with various immunological studies she was able to show that Monilia (Candida) like other organisms is susceptible to a logical classification and furthermore, she showed that nearly all, if not all pathogenic isolates, from whatever part of the body they were obtained, are one and the same species, *C. albicans*. Moreover, what differences (and there are definite differences) there are among various isolates are unworthy, in her opinion, of nomenclatorial recognition.

An especial advance was also made by Martin, Jones, Yao and Lee (179), whose clear-cut classification of the "medical monilias" reduced to 6, the 40 species of Castellani. This classification was later improved (178). Clear-cut evaluation of techniques and criteria are characteristic of both of these papers.

From 1935 on, the tendency has been to reduce the number of genera and species Ciferri and Redaelli (50) in 1935, reduced their six genera of 1929 (49) to four and in 1939 (51) to one, which they called *Mycotorula* Langeron and Guerra (150) reduced the six genera of Langeron and Talice (152) to one, *Candida* Castellani (39) still adhering to the generic name *Monilia*, reduced his forty species (36) to nine Only one important writer, C W Dodge in recent years, has recognized a multiplicity of genera and species

Dodge's book (70) "An Introduction to Medical Mycology" has classified Candida into an appalling list of genera and species The extent of Dodge's splitting may be deduced from the following considerations Eighty-two of the cultures sent in to Diddens and Lodder and reported in their book were diagnosed by them as *Candida albicans* Of these, 42 were labelled with Latin binomials, 10 different generic and 27 different specific names when they were received Many of these were authentic cultures from the laboratory where the name was coined Of these 42 isolates, (all *Candida albicans*, according to Diddens and and Lodder's diagnosis), Dodge recognized 14 species in seven genera! Of yeast-like fungi which produce blastospores and not arthrospores (*Candida*), Dodge recognizes over 100 species in 9 genera Dodge's taxonomic work with this group, it should be stated, is based largely on a careful study of the literature, not, so far as is indicated in the book at least, on actual laboratory studies Lest the reader think that the writer is unfair, he is referred to criticisms of this book by Henrici (109), Langeron and Guerra (150) and Diddens and Lodder (69) In spite of what has been said, it should be stated that Dodge's book is, and probably will be for many years, an immense help to any worker in the field The excessive splitting need not be accepted, and there is little to indicate that it is being accepted, but the excellent bibliographic data and the discussions of correct nomenclature are most helpful These are very extensive and obviously carefully prepared

MORPHOLOGY AND VARIATION OF CANDIDA

The morphology of Candida is studied somewhat differently than that of either the yeasts proper or the molds The usual method of growing molds on agar media and mounting in a mounting fluid, although sometimes used, is not very successful since the cells fall apart so readily The cultures are better examined directly on the petri plate, and also *in situ* after growth on cover glasses or slides, stained or unstained Many such methods have been used The method of Rivalier and Seydal (237) is probably most often employed, but Fortner's (96) and Soriano's (256) methods also have been used We use Henrici's (108, 253) method of growing them in agar media on cover glasses Of all the media used, the corn-meal agar of Benham (18) is in our hands the best to induce mycelial development In petri plates, if the agar is not too thin and the inoculation is made by scratching into the agar, pseudomycelium or true mycelium frequently will develop from isolates which show only yeast cells from surface inoculations One should examine young petri plate and cover slip cultures and follow them through for days or weeks to look for mycelium and blastospores

Potato decoctions (271) or the older method of gelatin stabs may be used This last method (249, 250), if properly employed, will usually demonstrate whether or not a mycelium is produced, but being a macroscopic method it gives no information as to the nature of the mycelium or other detail of morphology unless the gelatin tube culture is sectioned and examined, a not very satisfactory procedure Either glucose-peptone (so-called Sabouraud) agar or beer-wort agar or both and various liquid media also have been used for studying morphology On these liquid media the presence or absence of a pellicle may be of diagnostic value

Detailed study of the morphology of the Candidoideae, for the most part Candida, for practical purposes begins with the papers of Ciferri and Redaelli (49) The excellent paper of Langeron and Talice (152) soon followed, and this paper and the more recent articles of Langeron and Guerra (150) should be consulted for details of morphology In the latter publication the extent of variation within the best known species is clearly indicated with a full discussion and a series of excellent photographs This work although entirely original, recapitulates and expands much previous work (e g , 2, 84, 149, 179, 292) It is clear that although there are various ways in which the blastospores are borne from the mycelium or the pseudomycelium, in compact spherical masses, in loose verticals, as single scattered buds, etc , from the ends or sides of cells, or in clusters from morphologically distinct sporophores, all these and certain other types of blastospore production, may be found in the same species The justification for placing different morphological types in the same species rests on several factors Immunological reactions would place the same morphological type in different serological groups and different types in the same group Careful examination of the same culture on different media or under different conditions have revealed different morphological types The same culture on the same medium at different times may produce a different morphology Especially, a culture may dissociate into variants of considerably different morphology (see later section)

In mycology physiological characters are not much used in classification It is apparent that for yeasts they are almost the only characters that have value in determining species Moreover, as will be shown, physiological characters are stable Isolates in culture undergo morphologic degeneration or dissociation to a remarkable degree, but the fermentative characters remain practically unchanged It thus happens that the yeasts have for the great part been studied either by bacteriologists or by specialists recently designated by the more or less unneccessary term, zymologists

The practice of grouping together in a single genus or species organisms of similar physiology and showing in at least the lower titers an immunological homogeneity but of considerable difference in morphology may be somewhat startling to botanists Among bacteriologists it is taken for granted It is not that most bacteriologists necessarily accept in any way the theory of pleomorphism as applied to bacteria if the word is used in its original sense as the Tulasne brothers and DeBary used it They do realize that the same strain under differ-

ent conditions or different strains of a species under the same conditions will show a certain amount of morphologic variation Also bacteria in their youth, maturity, and old age will vary as individuals or as populations Langeron and Guerra (150) seem to be correct in maintaining that Candida is essentially a unicellular organism as are bacteria They minimize the true mycelium in the genus which at best does not predominate, and which is formed only under certain conditions Cells of Candida are individuals, and the position of cells (blastospores or cells in the pseudomycelium) manifests itself as "morphology" of the aggregate Thus differences in conditions may be manifested by different rates of this or that type of cell production and this will appear as a different morphology of the group A slight individual difference in single individuals may appear much exaggerated in the aggregate of cells For instance, two strains alike, except in the angle at which blastospores are sprouted from the pseudomycelium, will produce two different "genera" of Ciferri and Redaelli or Langeron and Talice Unless the differences in morphology of isolates which are often only differences in arrangement of fundamentally similar independent cells in a population, are correlated with habitat or with pathogenicity, there seems to be no practical reason for giving the isolates nomenclatorial distinction, and unless these differences are correlated with other morphological, physiological or immunological characters, there is no academic reason to recognize them as of taxonomic significance This is essentially the attitude of most recent workers on yeast-like fungi It was the attitude of Henrici (108) in 1930, who since he did not have the more recent data, probably carried it to an extreme, and of Benham who for practical purposes started the modern approach to classification of Candida, carried on so successfully by Martin *et al* (178, 179), Langeron and Guerra (150), Diddens and Lodder (69), and Mackinnon (168)

Variation It is evident that some of the Candidoideae are extremely "variable" Often this variability is merely a modification of type of growth under different conditions When the culture is replaced under the original conditions the initial type of growth eventually returns In addition, there are more permanent changes in morphology, either a seemingly gradual but nearly always irreversible change which we shall call "degeneration" after the nomenclature of the German dermatologists, or a sudden change usually called "dissociation" For a general discussion on variations in fungi, Henrici's (253) posthumously published chapter may be consulted

Reversible variations due to changed cultural conditions in Candida have been much studied By 1930 Henrici (108) stated that there had been "considerable investigation" Since then a number of papers have been published Mostly they refer to *Candida albicans*, but the same principles probably hold true also for other species In general, it has been found that where conditions are favorable for rapid multiplication, as with easily assimilated or fermentable carbohydrates and with abundant aeration, the unicellular yeast forms predominate Reduced oxygen tension, starvation media (such as Benham's corn-meal agar, potato infusion, or gelatin stab cultures), liquid media in general, which provide partial anaerobiosis below the surface, high pH, high temperature (37 C), etc ,

all tend to produce the mycelial growth of Candida. Perhaps the most complete study and review of these variations is that of Talice (271), who justly pointed out that much of the seemingly contradictory findings of different workers was due to the fact that different species had been used. Mackinnon (168) also points out that comparable results are to be expected only if the same R or S phase is used.

The first paper (197) on dissociation in Candida appeared as late as 1935, although a few papers on dissociation in non-filamentous yeasts had already appeared. Negroni by the treatment of cultures of *C. albicans* with immune serum induced a variant which he called "R". Rough, it undoubtedly was, but following the usage of Mackinnon (166–168) we shall not consider it homologous to the "R" forms of bacteria. The variant culture formed slow-growing colonies with irregular edge and rough surface. The variant was not pathogenic to rabbits. It had less tendency to form mycelium than the original culture. Similar results were obtained later (209) by the action of dyes on the normal *C. albicans*, and these results were later confirmed by Mackinnon (166, 167) who also got the same variants in old cultures on Sabouraud agar. The variants were termed "lethal" by Mackinnon because their slow growth and tendency to die in culture, unless transferred frequently, would inevitably tend to eliminate them. There were various degrees of this dissociation. Changes were almost entirely irreversible. Regressions were rare in Negroni's (201) experiments and only partial. A lethal variant was sent to the author in 1946 from Montevideo by Mackinnon. All the properties attributed to the variant in 1939 and 1940 still remained. The culture finally died when it was not transferred for a period of 5 weeks.

Mackinnon (166–168) reported on another type of variation which seems to have more in common with the microbic dissociation in bacteria. Starting with single cells of *C. albicans*, he obtained sectors of forms called at first M (formas membranosas) but really comparable to the R forms of bacterial cultures. These R variants have a tendency for a more extensive mycelial development at the expense of the blastospores. The smooth surface growth of yeast cells also tends to become replaced by elongated pseudomycelial cells. This causes the colonies to take on an irregular and filamentous edge. Whereas the normal or S forms produce no pellicle in liquid media, these R forms form a heavy pellicle. Virulence for rabbits is reduced to a point where 0.5 to 1 billion blastospores of a fresh young culture do not kill in 10 days but produce minor kidney lesions (lesiones de eliminación). Like the lethal variants, the R variants once dissociated, rarely revert, but if they do revert, it is only in part, and these partially reverted strains soon become rough again. Mackinnon has observed only one permanent partial reversion in eleven years of careful study.

Mackinnon noted with great clarity, that dissociations of the lethal and R types were different. He also showed that each type varied as to degree and thus he spoke of "directions of variations". For example, an organism may have dissociated a long or a short way in one direction and a long or short way, or not at all in another. Therefore, one might expect great morphological "variation"

in different cultures, all descending originally from the same single cell culture and all due to phenomena familiar to bacteriologists. The real reason for much of the confusion in the taxonomy of Candida becomes evident.

Confirmations of variations in the R direction have been published. Cavallero (43) induced variations, some permanent, and some which reverted at once to the original S form. Many dissociated only part way in the R direction. There is a full bibliography on variations in yeasts and yeast-like fungi in Cavallero's paper. A paper by Redaelli, Ciferri, and Cavallero (233) which the writer has not read and which Mackinnon admits difficulty in interpreting, has been reviewed at length by both Diddens and Lodder (69) and Mackinnon (168). Apparently these Italian workers dealt in part with reversible variations and in part with real dissociation, but the two phenomena were confused and, according to Mackinnon, the technique was inadequate.

Mickle and Jones (185), Martin and Jones (178) and Conant (55) were able to confirm the results of Negroni and Mackinnon and offer some special points of interest. These workers did not start with old laboratory cultures which already had or may have started to undergo spontaneous variation but with strains freshly isolated from the human body. By use of immune serum or lithium chloride, variants were induced. Through the fortunate use of blood agar (a medium considered "unusual" by some workers in the field!) an easy differentiation of S and R forms and of an intermediate stellate form was accomplished. The S forms were pathogenic to rabbits, the intermediate forms required twice the dose to kill, but the R forms were nonpathogenic in doses ten times that used with the S forms. Clear differentiation of the forms by agglutination titres was not obtained and fermentation reactions were not affected except that the R forms fermented more slowly. Details of differences in colony appearance are given, on both blood agar and glucose-peptone agar, of the appearance of growth on glucose-peptone broth, and of microscopic differences on various agar media.

The above mentioned authors emphasize the fact that old laboratory cultures have often dissociated spontaneously almost beyond recognition. By plating on blood agar one can usually reisolate the S form from such cultures. These workers at Duke University have stressed freshly isolated cultures and thus their various publications will probably be of most value to the practical worker who usually is confronted with the problem of identifying a freshly isolated, rather than an old laboratory, culture. Possibly the discussions of Diddens and Lodder, based largely on laboratory cultures, often apparently in various stages of degeneration, will help him less. On the other hand, it is part of the value of these latter workers' discussions that they cover the whole range of morphologic variations within a species.

Papers by Castellani (40), Langeron and Guerra (151) and Besta (22) are not included in this discussion, because the observed natural or induced variations reverted so readily, and it is apparent that dissociation, as usually understood, is not involved. Papers, unfortunately not available, by Hollström (121, 122) apparently attempt to show that *Monilia Pinoyi* (*C. albicans*) or other fungi and the tubercle or leprosy bacillus are both stages of the life cycle of the same organ-

isms. Comment had better be reserved until the original reports have been consulted.

The S form of *Candida albicans* as it appears when first isolated from its normal habitat has usually been regarded as the normal form and in this the author agrees. He disagrees with the view that the R form for either bacteria or Eumycetes, as it finally occurs under artificial conditions of culture, is necessarily the culmination of the microbe's existence. One may consider induced variation in fungi an acceleration of a process which tends to take place spontaneously when they are maintained in culture. The word "degeneration" for this process seems most appropriate. *Candida* species are not the most extreme examples. The dermatophytes degenerate in culture to such an extent that in many cases only freshly isolated cultures may be diagnosed at all. It is hard to consider such irreversible changes in *Candida* or dermatophytes as anything other than a degeneration when we consider that the variation is in the direction of development of mycelial strands at the expense of reproductive cells. One can hardly consider a development other than a degeneration which, except in unnatural conditions of culture, would tend to eliminate the strain by reducing the chances of the organism to reproduce itself. The writer does not consider any explanation of variation in Candida depending upon non-sexual hyphal fusions (such as Brierley, Hansen, and others show are at least partially responsible for variations in certain molds) or upon sexual fusions of nuclei. Indications are that yeast cells are uninucleate and, so far as the writer knows, there is no evidence of a sexual fusion of cells of *Candida*.

Reported perfect stage of Candida. Earlier workers frequently attributed an ascogenous stage to "Monilia" and indeed often put species in the perfect genus *Endomyces*. Redaelli, Ciferri, and Cavallero (234) believe that chlamydospores had been diagnosed as asci. This is probably the explanation for the occasional classification of *C. albicans* in the genus *Endomyces*. It is true that some isolates diagnosed as other species of *Monilia*, *Candida*, etc., have been found (68, 69, 261) to form ascospores, and to be actually *Saccharomyces*, *Pichia*, etc. Some species of *Candida*, are probably imperfect stages of *Saccharomyces*, *Pichia*, *Endomycopsis*, etc. However, some species are completely and permanently (so far as can be determined) anascosporogenous. It is noteworthy that there is no real proof of sexuality in *C. albicans*, the organism on which most of the work on variations has been done. Although this species has been classified in *Endomyces*, *Endomycopsis*, and *Saccharomyces*, it is noteworthy that workers who have studied this species most intensively, have not found a perfect stage for *Candida albicans*.

The immunological reactions of Candida (2, 18, 177, 264, 265) will be discussed here only in so far as they have to do with classification. It has been established that fairly potent immune serums may be prepared in rabbits. Agglutinin, agglutinin absorption, precipitin and complement fixation tests are possible. Unfortunately, it has been shown that antigens are often common to definitely different species and that sometimes agglutination titers are as high for other

species as for different isolates of the same species To a less extent the same sort of thing is true for agglutinin absorption and complement fixation tests At best, immunological reactions only allow the placing of an isolate in one of four or five broad groups Since the immunological groups in many cases are not natural physiological, morphological, or ecological ones, not very much is gained by using immunological reactions in the taxonomy of Candida These conclusions are essentially those of many medically trained workers as well as others

PHYSIOLOGY OF CANDIDA CULTURAL AND BIOCHEMICAL CHARACTERS

The fermentation of sugars is of very great value in identifying species of *Candida* and *Brettanomyces* Like most fungi and many bacteria, Candida in the early days was classified exclusively on morphological characters Castellani in a series of publications (36) extending over many years used fermentation characters almost exclusively Partly due to different, often improper, interpretations of Castellani's work, partly to differences in techniques, and partly to the inherent weakness of his system, it has fallen into disregard Gradually a system retaining parts of Castellani's fermentation schemes, but correlating them with other physiological and morphological characters has been built up

For studying gas production in the alcoholic fermentation, Diddens and Lodder use the unwieldy Einhorn (Smith) tube, and Langeron and Guerra, and Martin and associates, a paraffin or petroleum jelly seal We sometimes use a mixture of the petroleum jelly and paraffin and find it better than either one alone In our laboratory, the Durham tube containing freshly prepared 1% peptone and 3% sugar in 30 ml amounts gives reproducible results as to gas production The incubation temperature is room temperature which in most North American laboratories is close to 25 C Most recent authors find gas production from sugars to be a valuable taxonomic tool (69, 84, 149, 168, 178, 179, 292)

Very important in this connection is the fact that observations on dissociation in Candida have indicated that however much the morphology varies, the outstanding cultural characters remain Sometimes the rate of fermentation is altered and rarely a fermentative character (e g , maltose fermentation) may seem to be lost, but the character soon returns Martin and associates (178, 179) have stressed the importance of proper standardization of technique in this regard We have found, like Benedek (16), that we could not confirm our own results when ordinary bacteriological methods, e g , small Durham tubes and 1% sugars were used But when higher percentages of sugars with the petroleum jelly-paraffin seal were used, the results were always reproducible We have also found that freshly prepared 3% sugar broths in large amounts in Durham tubes gave reproducible results, but we should recommend the Martin technique in case of doubt Wachiowak and associates (284) who, like Benedek, had not found the fermentation characters constant, also used a technique which is considered inadequate, 1% sugar and probably the Durham tube Croft and Black (62) suggest that the failure of a yeast to form gas in Durham tubes may be due

to mechanical blocking at the base of the gas trap, and therefore they recommend a paraffin seal We always shake the culture gently after 24 and 48 hours of incubation to prevent this blocking

Recent biochemical studies (145, 210a) have shown that the results obtained by the use of bacteriological fermentation tubes are not always obtained when more precise fermentation methods are employed A bacteriological fermentation tube may show little or no evidence that a sugar is fermented, but the manometric tests may show a definite anaerobic production of CO_2 by the same isolate For the present, at least, our taxonomic criteria must be based on the less exact bacteriological fermentation tube It possibly behooves taxonomists, however, to avoid unqualified dogmatic statements that this or that sugar is or is not fermented by this or that species of yeast

The determination of availability of various organic substances, especially sugars as the sole source of carbon and certain nitrogenous substances as the sole nitrogen source, e g , nitrates, asparagine, urea, etc , is second in importance to fermentation studies A recent paper by Wickerham (290) throws a definite doubt on the value, or at least the meaning, of such studies It appears that in these studies the ability of the organism to metabolize the nitrogenous compound is really the ability of the organism to metabolize the compound in the absence of or with a minimum of growth promoting substances Nickerson's (210) observation with Zygosaccharomyces that organisms which utilize nitrate, also reduce it to nitrite, should be investigated further to see if this phenomenon extends to Candida More clear-cut results would be expected by determining the presence or absence of nitrites than by determining availability of nitrates by the auxanographic or other methods

Biochemical Investigations Two important papers (145, 210a) have been published on the biochemistry of the fermentation of sugars by Candida Except for this there has been little work on the physiology of Candida The yeasts proper have been so thoroughly studied that one might have expected that more would have been written about the filamentous yeasts Moreover, what work that has been done is scattered among papers on yeasts proper For this reason it is probable that much information actually available has been missed So far as the writer knows, no attempt has ever been made to use Candida to produce alcohol commercially, nor have they been used as leavening agents There are possibilities here, it would seem, since some isolates grow and produce gas very rapidly The author doubts that Custers' (64) statement that "Candida strains ferment weakly or not at all" will be substantiated for all species when the biochemical work necessary to prove this statement has been done An *Endomycopsis* species is known to produce amylase (291), and the writer has seen isolates of *Candida* which do the same If an organism can be found, that can produce alcohol from starch without malting and is more efficient than the Mucors sometimes used for the purpose, it may possibly find use in an important industrial process

Pringsheim (227) noted the production of amyl alcohol by species of *Monilia*, and "*Monilia formosa*" was found (245) to produce 1-ethylene oxide-α, β-dicar-

boxylic acid. Yukawa (302) found the reason for the presence of *p*-hydroxyphenyl lactic acid in koji but not in shoyu was that the organisms (Monilia and Mycoderma) which produce this substance from tyrosine are present in the former product but not in the latter. Species of both these genera produce this compound from tyrosine. Monilia also produced *p*-hydroxyphenyl ethyl alcohol but not tyramine from tyrosine. Prescott and Dunn (226) state that at least one Monilia produces lactic acid. The dearth of literature on industrial uses of Candida may be appreciated from the fact that the above well documented book lists (in the index, at least) only this one reference to *Monilia* (medical sense) or *Candida*. Smith's "Industrial Mycology" (1946 ed.) lists not a single reference except "*Monilia sitophila*" which very definitely is in no way a yeastlike organism.

A yeast, probably a Candida, has been used in the Soviet Union (223, 252) for producing cattle feed. Seaweeds, corncobs, straw and other pentosan-containing substrates are used together with added inorganic nitrogen. These are metabolized into yeast proteins of high nutritional value, much as *Cryptococcus utilis* with starch, rather than pentosans as a carbon source, has been used in western countries. Pentoses are not commonly fermented by yeasts. Lechner (154) used "*Monilia candida*" for studying protein synthesis from inorganic nitrogen. He found that the protein yield was slightly lower in the yeasts growing on glucose than in those growing on xylose. Two molecules of the sugars are split off and the rest go into protein synthesis. The xylose is completely utilized. The velocity of protein synthesis is more rapid with galactose than with xylose, and it is still more rapid with glucose. The hexoses and pentoses of sulphite liquors, especially those of beechwood are efficiently utilized, and the action goes on best if the liquor is kept slightly acid. Enebo and associates (77) have found that the protein content of a variety of *C. pseudotropicalis* (*Torula lactosa*) was uniform regardless of the conditions of growth.

True yeasts and molds have been used to synthesize fats commercially. *Endomyces vernalis* a perfect yeastlike fungus probably more closely related to *Trichosporon* than to *Candida* has been extensively used. Haeseler and Fink (100) and Heide (107) have reviewed the subject. Peck and Hauser (215, 216), in a detailed study of the lipids of *C. albicans*, found 5.3% of the dry weight of the cells to consist of these substances. Of these, 97% was acetone-soluble, the rest were phosphatides. In the acetone-soluble portion, glycerides, free fatty acids and free and bound sterols were identified. Several common fatty acids were identified. Rippel (236) found that *Nectaromyces* (*Candida*) *Reukaufii* produced 10 to 15 parts of lipid per 100 parts of carbohydrate consumed. All workers have found that media which promote the best growth are inferior for lipid production. The fairly large literature on lipid production by yeasts has been surveyed by Starkey (260). Tauson (275) is said to have found that species of *Monilia* were able to assimilate paraffin wax. Fouts (88) has studied the lipolytic action of "*Myco lipolytica*", probably *Mycotorula lipolytica* i.e., *Candida lipolytica* was meant. Others have noted the fat-destroying ability of this species, but have not studied it extensively.

Little is known of the exact nutritional requirements of species of *Candida* Rogosa (243) has found that only lactose-fermenting yeasts require an exogenous source of nicotinic acid Among the lactose-fermenting yeasts listed were *Monilia pseudotropicalis*, *Mycotorula lactis* and *Torula lactosa* According to Diddens and Lodder the first two are *Candida pseudotropicalis* and the last *C pseudotropicalis* var *lactosa* Burkholder and associates (28, 29, 30) have made an extensive study of the biotin, pyroxidine, pantothenic acid, niacin, inositol, and thiamine requirements of 18 species of *Candida* Burkholder (27) has also studied the synthesis of riboflavin by yeasts, including "*Candida Guilliermondia*" (*C Guilliermondii*) He found that mechanical agitation during growth favored the synthesis A large initial inoculation is unfavorable for riboflavin production It is interesting that arabinose, galactose, inulin, maltose, mannitol, sorbitol or xylose produces good growth but little riboflavin while a sugar that is fermented, namely fructose, glucose, mannose or sucrose produces equally good growth but also good riboflavin synthesis Galactose added to glucose media increases growth but cuts down synthesis of riboflavin Van Lanen (281) has shown that *Candida Guilliermondii* and "*Torulopsis lactosa*" (probably a variety of *C pseudotropicalis*) absorbed from an unfortified medium an unusually large amount of niacin Almquist (3) lists "*Monilia albicans*" and "*Monilia candida*" among the organisms not able to synthesize vitamin K Landy and Dicken (148) find that only very minute amounts of biotin are synthesized in biotin-free media by *C albicans*

As was stated earlier, there has been considerable work on the effect of nutritional and physical changes on the morphology of Candida These studies as well as many of the cultural studies are more or less unsatisfactory from the biochemical viewpoint A few more precise experiments have been performed Talice (272) has studied the effect of pH on *C albicans* Unlike the dermatophytes, *C albicans* grew over the same wide pH range, 2 2 to 9 6, as did saprophytic molds and yeasts The optimum was pH 7 0 The acid tolerance is of advantage culturally The ordinary Sabouraud agar need not be used for isolation, but acid media in which bacteria will not grow may be used This facilitates isolation of Candida from habitats in which bacteria outnumber the yeast Berry and Magoon (21) found a species of *Monilia*, obviously *Candida*, that grew sufficiently at −4 C to produce a definite pellicle on liquid media *C albicans* was found to have good growth at 18, 22, 37, and 45 C, but a slightly delayed growth at 45 C (303) Beamer and Tanner (13) studied the heat resistance of "*Monilia candida*" It was found to be more heat resistant in acid media and the rate of death was logarithmic in acid media and in sterilized grape juice Kadisch (136) found that *Candida albicans* is destroyed at about the same temperature as molds, 60 C for 10 minutes is usually sufficient if the cells are moist When the cells are relatively dry they are more resistant than the molds tested It has been shown (217) that *C Krusei* has considerable influence on the heat resistance of streptococci growing in the same culture The relative humidity of the air had little or no influence on the gross morphology or rate of growth of "*Candida candida*" (295)

At least two writers (90, 147) have attributed the ability to fix atmospheric nitrogen to yeast-like organisms Considering the fact that so many past claims of nitrogen fixation by microörganisms have been disproved and that nitrogen fixing ability has been proved for relatively few organisms, we shall have to view these claims with reserve

TAXONOMY OF CANDIDA

Diddens and Lodder have recognized 25 species and 8 varieties of *Candida* It should be stated that many of these species are rare in the habitat usually studied, namely the human body The fact that the species are rare or unknown in a particular habitat should in no way preclude their being accepted as "good" species This point is emphasized as there seems to be a tendency among some workers to deny or to ignore the existence of such species The fact that well over 90% of the work on Candida has been done on isolates from the human body probably explains why some species are considered rare If Candida is searched for it will be found in many different habitats This point will be stressed later

If classifiers must be classified as either "lumpers" or "splitters", Diddens and Lodder will have to be called lumpers, although they have not carried their lumping to an extreme Henrici (253) has complained that in the field of mycology there is a dearth of lumpers and a plethora of splitters Possibly the complaint is less justified now than it was when he wrote his comment in 1939 It is to be hoped that future workers on Candida will resist the inclination to create new species unless the necessity for such creation is very great

Candida albicans This species has a few outstanding characteristics Morphologically, the most obvious is the production of chlamydospores Chlamydospores are round (or nearly so) heavily walled cells usually intercalary in most fungi, but in this species usually terminal In fresh isolates these are nearly always a prominent part of the morphology and in older cultures they may usually be found in proper media if they are searched for Martin and Jones (178) found all 233 of their freshly isolated cultures produced chlamydospores Benham's corn (maize) meal agar, not the sugared medium of the phytopathologists, is our medium of choice, although most non-North American workers seem to prefer potato infusion (271) or potato agar Some difficulty may be experienced in getting a clear corn meal agar The writer has used a method essentially that of Bernhardt (20) It would appear that any method of preparation involving high temperatures for extraction (1) would give a medium very different in composition from Benham's original one The not inconsiderable enzymatic action in the infusing corn meal is prevented by high temperatures Yellow corn, not light colored as the author has erroneously stated (253), is preferred Recently we have used Anderson's (5a) corn meal infusion Although less convenient than corn meal agar, this medium seems to have some advantages for inducing production of mycelium and chlamydospores On either the corn meal agar or broth, the true mycelium, whose formation Wickerham and Rettger (292) have so carefully followed, is very apparent

Except for the chlamydospores there is little other morphological detail that will set a strain of *C. albicans* apart from other species. Freshly isolated cultures show little tendency to formation of true or pseudomycelium unless grown in starvation media below the surface, as in scratch corn meal agar plates or potato infusion broth, or in sugar-free beef peptone gelatin stabs. Grown on ordinary Sabouraud agar the cells are almost exclusively of the budding yeast type. Strands of mycelium may penetrate into the substrate after prolonged incubation but they are much more numerous and appear more promptly along the scratch in corn meal agar. Blastospores are invariably produced from the strands but the arrangement of blastospores varies so much between isolates that discussion of this has little value in a review of this sort. They tend to occur in ball-like clusters in fresh isolates, but not to the extent that they do in *Candida albicans* var. *stellatoidea*. See discussions and figures of Wickerham and Rettger (292), Fisher and Arnold (84), Langeron and Guerra (150), and Martin and associates (179).

Candida albicans is the only species which ferments (gas production) glucose, galactose and maltose but not lactose or sucrose. Under some conditions, with some strains, smaller or larger amounts of gas from sucrose will be formed but acid is typically produced by all strains. See Kluyver and Custers' (145), and van Niel and Cohen's (210a) biochemical experiments for explanation of this. Strains have been found to lose temporarily their maltose-fermenting ability (168). It is well to recall that the dissociated R cultures have all the important biochemical characteristics of freshly isolated smooth cultures.

A word about *Candida albicans* var. *stellatoidea*. Isolates of *Candida* differing slightly from *C. albicans* have been described (131, 178). They have been called *Candida* (or *Monilia*) *stellatoidea*. The formation of stellate colonies with a green zone of methemoglobin around them in ordinary blood agar (132), the lack of ability to utilize sucrose (168), and the lack of pathogenicity for animals shows that they are a more or less distinct entity. The many points of similarity including chlamydospore production, probably justified Diddens and Lodder in giving nomenclatorial recognition (as a variety) to these organisms. Some authors do not recognize a difference between the species and the variety, and it must be confessed that the difference is slight, but without ever actually studying *C. albicans* var. *stellatoidea*, the writer will venture that they are distinct. It is interesting that this variety has recently (183) been shown to be slightly pathogenic to chick embryo.

Candida Krusei. This species is somewhat different from most of the other species of the genus. It grows in liquid media largely as a heavy pellicle. The mycelium is entirely a pseudomycelium, never a true mycelium as sometimes is found in *C. albicans*, for instance. Mackinnon and Artagaveytia-Allende (169, 171) have called attention to the fact that this species is very like, probably identical with, *Mycoderma cerevisiae* and indeed Mackinnon (168) intimated that if he had not had medical training he would have diagnosed his cultures of *C. Krusei* as a species of *Mycoderma*. Again the distinction between the two subfamilies of the Cryptococcaceae is difficult to maintain. It has been found

that the perfect stage of many species of *Candida* is *Saccharomyces* (68), but it is evident that the perfect stage of *C Krusei* and of *Mycoderma* would be Pichia The possibility of *C Krusei* being the dissociated stage of *Mycoderma* should be investigated The fact that most workers in yeasts other than medical workers are unfamiliar with *C Krusei* and that most medical workers are at least as unfamiliar with *Mycoderma* and *Pichia* makes it appear very probable that very much of the past literature in the two fields may have been dealing with the same organisms As a matter of fact, much of the older non-medical as well as the medical literature on *Mycoderma* assumed the filamentous nature of the genus Among medical men this assumption was so usual that Vuillemin and Benham placed such pellicle formers as *Geotrichum* (*Oidium lactis*) in the genus *Mycoderma* According to Lodder's (159) argument, which seems sound, this latter concept of many French and North American workers is incorrect Stovall and Bubolz (265) had also considered *C Krusei* as a *Mycoderma*, separating this species from the rest of the "monilias" The writer (253) has previously suggested the possibility that some film forming yeasts with pseudomycelium might be grouped with *Mycoderma*

Candida Flareri This species was relegated to *C intermedia* by Diddens and Lodder and to *C parakrusei* by Conant (55) and Martin and Jones (178) These studies were thought by Mackinnon to be based on improperly labelled cultures since the original description of *C Flareri* showed no fermentations and both the above mentioned studies showed fermentation of sugars A study of a strain identical in properties to the original is of some interest Mackinnon and Artagaveytia-Allende (170) isolated it as a single colony from many colonies of *Rhodotorula mucilaginosa* All the properties of the two species were exactly alike, except that the *C Flareri* lacked the pigment of the Rhodotorula Mackinnon (168) is of the opinion that *C Flareri* is merely a colorless variant of *Rhodotorula mucilaginosa* Identical morphological and biochemical properties make this an interesting possibility and again indicates the varied phylogeny of *Candida* *Rhodotorula* seems clearly derived from the Basidiomycetes through *Sporobolomyces*, not from the Ascomycetes as we assume for most species of *Candida* See Lodder (159), Diddens and Lodder (69) and Henrici (111) for justification of this statement *Rhodotorula* must be clearly distinguished from the distinctly different *Cryptococcus pulcherrimus*

Candida tropicalis This is the only species of *Candida* other than *C albicans* that Mackinnon found to be pathogenic to laboratory animals Large numbers of cells (500 million blastospores) did not kill rabbits but produced minor lesions with or without abscesses near the renal pelvis Subcutaneous inoculations produced lesions only in the uriniferous tubules and these lesions were soon healed In the mouse, death was produced by 180 million cells in 1 to 10 days In the rat 500 million cells introduced intraperitoneally, produced renal abscesses and myocarditis with death in 1 to 10 days Half as many cells killed only rarely In the rat, intratesticular inoculations produced caseous lesions but the infections did not generalize Under all the above conditions, inoculations with *C albicans* killed the animals before or after a generalization of the

infection Infections of the joints have been produced in guinea pigs by inoculation with cultures of *C tropicalis* (182) Stovall and Pessin (267) had also found *C tropicalis* to be slightly pathogenic to rabbits if very large doses were given, and Meyer and Ordal (183) found that this species was capable of causing in the chick embryo an infection which was sometimes fatal It is evident that *C tropicalis* is an organism of very low virulence for laboratory animals How important it is in human infections is difficult to say One might venture the suggestion that infections of human beings are relatively rare and unimportant It is evident that *C tropicalis* is frequently isolated from the human body as well as from other habitats The difficulty of proving that infections are actually due to organisms so widely distributed and of such low virulence is very great

Candida parakrusei (*C parapsilosis,* according to Diddens and Lodder) This is another species that will have to be considered as capable, though rarely, of human infection Five cases (91, 129, 225, 293, 294) of endocarditis in which the only organisms isolated from the blood stream were *C parakrusei* (4 cases), and *C Guilliermondii* (one case), have been recorded, and the possibility of the organisms being nonpathogenic or contaminants seems very slight indeed They are pathogenic in the sense that viridans streptococci are pathogenic All these five cases were of patients who were drug addicts These differ from a recent case (289) in which *C albicans* was repeatedly isolated from the blood stream, in that this one did not end fatally, and in that the patient was not a drug addict The writer does not have enough data to venture an opinion as to whether Diddens and Lodder were justified in bypassing the earlier and more familiar specific name *parakrusei* for *parapsilosis* Mackinnon (168) has published excellent photographs showing that cells very like, if not actually, arthrospores are sometimes produced by this species Indeed, Langeron and Talice (152) had put isolates of this species in their genus *Geotrichoides*, a later synonym of *Trichosporon,* which produces both blastospores and arthrospores

Candida humicola Another species, *Candida suaveolens,* which Diddens and Lodder show should be termed *C humicola,* also is said to produce arthrospores Ciferri and Redaelli who called the organism a Geotrichum, Magalhães (173) who used this species as the type of a new genus, Puntoni (229) who called it a Trichosporon, all found arthrospores Both Mackinnon (168) who studied Magalhães' culture, and Diddens and Lodder, who studied a culture sent to them by Langeron, who got it from Ciferri, have classified this species as *Candida* in spite of the recorded presence of arthrospores Diddens and Lodder believe that mixed cultures have been involved, possibly a Geotrichum and *C humicola* It is the opinion of the writer that the ability to produce arthrospores, may, in some isolates, be lost in cultivation About a year ago he isolated from soil, a Trichosporon which produced copious arthrospores as well as few typical blastospores from the same hypha After only a year's incubation it has completely lost its ability to produce arthrospores The mycelium is more mold-like and the septa are so scarce that one can hardly designate the elongated cells as arthrospores The ability to form blastospores to some extent

still remains In other words, the culture has degenerated according to the pattern which Henrici (253) has described It is thus possible that mixed cultures or contaminations were not involved in the confusion of the nomenclature of *C suaveolens* but rather a spontaneous degeneration of the culture This confusion incidentally is much more involved than is here indicated

Mackinnon (168) was unable to demonstrate any evidence that "*Candida suaveolens*" was pathogenic to laboratory animals although Nottebohm and Negroni (212) isolated cultures from an infection of the lip The organism was obviously either the causative agent or a secondary invader, for yeastlike cells were numerous in direct smear preparations A consideration of the morphology of the two last mentioned species of *Candida* indicates that the boundary between *Candida* and *Trichosporon* is not a sharp one It must be recalled that *Candida* is a "form genus" of the Fungi Imperfecti, which is admittedly used for convenience and like other form genera may not be a phylogenetic unit

Candida pulcherrima The well known species of yeast *Cryptococcus pulcherrimus* (Lind) Benham, as it grows usually has little or no pseudomycelium and is usually classified as a Torula, Cryptococcus, or Torulopsis It shows a tendency to dissociation (degeneration) in cultures, and R forms produce a pseudomycelium (228, 238) On this basis Diddens and Lodder transferred this species to the genus *Candida* Lodder (159, 160) had already built up a good case for this being the type species of the genus *Torulopsis* Since a type species should not be transferred to a later genus without changing the name of that genus to the earlier name, *Candida* would have to yield to *Torulopsis* Another name would then have to be found for "*Torulopsis*" or the genus be retyped However, this need not concern us too much *Candida* will probably be validated as a *nomen conservandum* at an early Botanical Congress (and in the meantime almost everybody is using it) and if it is not validated, *Syringospora* still has priority over *Torulopsis* Besides, the writer is not convinced that the transfer ought to be made It is true that variants produce a pseudomycelium, and laboratory cultures frequently show this, but so do many other yeasts form a pseudomycelium in the same way Indeed the writer will venture to agree with Henrici (111) who stated that one will find a mycelium in most yeasts if he studies them sufficiently This is one of the borderline cases between the two subfamilies of the Cryptococcaceae that was mentioned as giving Diddens and Lodder some difficulty Actually where one taxonomic entity leaves off and another begins will probably always give some difficulty This is the expected state of things in taxonomy, because nature has not classified organisms with the definiteness, certainty, and permanence that scientists with limited taxonomic experience seem to expect of systematists

To confuse the question of the proper classification of *Cryptococcus pulcherrimus* is the fact that sexual spores have been attributed to this organism The writer has searched diligently to find them in two cultures but without success However, the combined work of Windisch (296) and Roberts (238), must convince one that they are to be found in some isolates at least Since the usual form encountered is the imperfect stage (ascospores are formed only sparingly,

belatedly, and only in some isolates) and since it is a common practice to classify the asexual stage in the Fungi Imperfecti separately from the perfect stage (e g, *Fusarium* or *Cladosporium*, each of which has a perfect stage bearing another generic name), it will probably be advantageous to classify the yeast in question for convenience, if for no other reason, as a Cryptococcus or as a Torulopsis if the latter generic name is favored.

ECOLOGY OF CANDIDA

It has been stated that species of *Candida* have usually been isolated from the human body One would almost believe that they were found exclusively there from a cursory study of the literature, and indeed some medical writers seem to assume this Actually there have been many scattered isolations from a "nonparasitic" habitat to use Diddens and Lodder's terminology, but these isolates have often been so poorly described that, until Diddens and Lodder made the diagnoses, one would hesitate to place many of them in a genus, much less in a species Most nonmedical workers have ignored the yeastlike fungi Usually they were not found, or if found, no attempt was made to identify them As an example, we may take the habitat, soil Gilman (94) lists 169 authors who have recorded one or more named species of Eumycetes isolated from soil Eight species are put in the genus "*Monilia*" None of these seems to belong to Candida "*Monilia candida*", however, was found in soil by Adametz in 1886, but what genus or what species was meant, it would be difficult to determine Only two of Diddens and Lodder's large collection were isolated from soil One might assume from this that Candida is very rare in soil However, during the past few months a student of mine, Mr R Bouthilet, has made a survey of local soils for yeasts and has readily isolated several cultures of *Candida* An occasional reference to Candida in dairy products will be found in the literature, but a very large literature on non-filamentous yeasts is in existence Recent indications are that Candidas are very numerous in these products even sometimes outnumbering the nonmycelial yeasts Chinn and Nelson (47) found among 333 yeast cultures from cream and butter, 197 Candida, 3 Saccharomyces, 41 Rhodotorula, 81 Cryptococcus, and 11 Trichosporon Stacey (258) found "Monilia" to be the principal cause of spoilage of soft cheese The writer has frequently isolated Candida from imported and domestic Camembert cheese It is all but impossible to believe that Candida was absent from all the thousands of samples of soil and dairy products examined by previous workers More likely, lack of interest in, or lack of familiarity with Candida accounts for the curious paucity of records on the subject

Recently the distribution of *Candida* species on fruits or vegetables, sound or undergoing decomposition, has been studied Although these habitats have had extensive study by professional mycologists, Candida has until recently rarely been recorded there However, Mrak and associates found that out of 241 cultures of yeasts isolated from grapes or grape products (188), 18 were Candida, mostly *C Krusei* Nine of the 67 cultures of yeasts from Californian and Egyptian dates (189) were Candida, five *C Chalmersii*, two *C tropicalis*,

and one *C Krusei* From 115 cultures of yeasts isolated from souring figs (190) 26 were *C Krusei*, and 6 *C Chalmersii* The writer is unable to decide on the proper name for *C Chalmersii* Mackinnon, and Langeron and Guerra accept this as a good species, but Martin and Jones as well as Diddens and Lodder do not recognize differences between this and the organism that the former call *C parakrusei* and the latter *C parapsilosis*

Negroni and Fischer (208) indicate that *Candida* species are fairly abundant in decomposing fruits and vegetables They made an examination of 30 samples from the delta region of the Paraná River in Argentina, and isolated and identified 5 species from 4 samples *C Krusei* (3 times), *C Chalmersii*, *C tropicalis*, *C paranensis* (a new species close to *C Guilliermondii*) and *C Aldoi* They isolated also 8 other imperfect yeasts and 62 other fungi The most likely explanation for the failure of most previous investigators to record this habitat for Candida, is that this genus was not searched for, not that it was absent Negroni's finding *C Aldoi* is of some special interest According to Diddens and Lodder and others this species is synonymous with *C albicans* If this be true and if the diagnosis was correct, which we can be certain is the case since it was made by Negroni, this would be the first authentic record, so far as the writer knows, of *C albicans* being found in a nonparasitic habitat It is to be hoped that this will be investigated further, and that animal inoculations will be made with this isolate

Diddens and Lodder recorded the original source, where it was possible, of all the cultures which they studied Most of them were isolated from the human body The following lists cultures not from this source The figure represents the number of cultures, the source is enclosed in parentheses

1 *Candida albicans* None of the 79 strains was from a source other than the human body, two of them recorded as occurring elsewhere (232, 283) were diagnosed as other species by Diddens and Lodder
2 *C Krusei* 1 (apple, Greece), 3 (bakery yeast, Belgium, Portugal), 2 (compressed yeast, Netherlands), 2 (lambic beer, Belgium), 1 (fermenting cacao, W Africa), 2 (palm wine, W Africa), 1 (fresh mash from potato starch factory, Netherlands), 1 (Raulin's solution, France), 5 (salt pickles, Netherlands)
3 *C tropicalis* 1 (distillery yeast, Germany), 2 ("*Torula utilis*" feed yeast, Germany), 1 ("*Torula utilis*" compressed yeast, Germany), 1 (kefyr milk, Netherlands), 1 (sauerkraut, Germany), 2 (tea fungus, Java), 1 (lambic beer, Belgium), 1 (insect larva, Germany)
4 *C pseudotropicalis* 2 (dairy sewage, England), 1 (buttermilk, Netherlands), 1 (raw milk, Germany)
5 *C Guilliermondii* 2 (flowers, Germany), 1 (buttermilk, Netherlands), 1 (bottled wine, Germany), 1 (air, China), 1 (decomposing carrots, Italy), 1 (scale insect blood, India), 1 (distillery yeast deposit, Italy), 1 (gooseberry jelly, Germany), 1 (tobacco, England)
6 *C mycoderma* 1 (flower, Germany), 1 (brewery yeast, Netherlands), 1 (cheese, Netherlands)
7 *C zeylanoides* 1 (beef, Australia)
8 *C parapsilosis* 2 (tea fungus, Java)
9 *C pelliculosa* 1 (lager beer, Netherlands)
10 *C lipolytica* 1 (rancid margarine, Netherlands)

11 *C Melinii* 7 (wood pulp, Sweden), 1 (pine heartwood, Sweden), 1 (bark beetle, United States)
12 *C tenuis* 3 (bark beetle, United States)
13. *C Scottii* 1 (beef, Australia), 1 (soil near "meatworks", Australia), 1 (wood pulp, Sweden)
14 *C robusta* 1 (buttermilk, Netherlands)
15 *C macedonensis* 1 (compressed yeast, Netherlands)
16 *C Reukaufii* 1 (flower, Germany) Many cultures of this species under the name of *Nectaromyces Reukaufii* have been found in insects and in nectar
17 *C humicola* 1 (peat, Switzerland), 1 (exudate from yew tree, Germany), 1 (corn mash, Dominican Republic)
18 *Candida pulcherrima* (*Cryptococcus pulcherrimus*) 2 (apple skin, Luxembourg), 1 (fruit, Switzerland), 2 (grapes, United States), 1 (dates, Germany), 3 (cherries, Germany, Switzerland)
19–25 The following species represented in Diddens and Lodder's collection by three or fewer species each, none of them of known non human origin With so few records one would hardly risk stating that their only, or even principal, habitat was the human body *Candida heveanensis, C intermedia, C Brumptii, C catenula, C mesenterica, C rugosa, C japonica*

Besides these habitats represented by cultures, in the Delft-Baarn collection, there have been scattered references in the nonmedical literature to Candida or Monilia tan liquor, France (45), pathogenic on insect which was parasitic on another insect, U S S R (276), causing bluing of macaroni, U S S R (81), causing "mouse disease" of wine, U S S R (46), cacao, Puerto Rico (48), chilled beef, where some species grow slowly below 0 C, Australia (76), souring figs and dates, U S A (189, 190), paper mill, Canada (97), chicha (maize beer), Argentina, Bolivia (168, 257), grapes, Uraguay (168), termites, India (128), wood pulp, Italy (95), rotting apple, fruit of palm, decomposing quince, domestic bread dough, Argentina (208), sour milk, U S A (27), cream and butter, U S A (47), grapes and grape products, U S A (188), cheese, Great Britain (258), insects, Germany (192), bark beetle, U S A (244)

There are probably other habitats for the genus published but the above will indicate the wide distribution of Candida

MONILIASIS

General considerations Moniliasis is a general name for any infection due to any species of *Candida* It is to be hoped that the tendency to change the name of a disease to follow taxonomic changes will not extend to this disease Thrush (or other oral moniliasis) is the best known form of the infection but others are known such as those of the nails or of the skin, about the mouth, under the arm pits, in the inframammary region, the groin, the hands and especially the feet Vaginitis, vulvitis and infections of the respiratory tract due to Candida are also important Infections involving the central nervous system and the heart are very rare, and intestinal moniliasis has been described, but its existence has been doubted It is probably unnecessary to recommend to readers the recent and excellent "Handbook of Medical Mycology" by Conant, Martin, Smith, Baker and Calloway (56) not only for the concise clinical discussion but the mycology as well The older discussion of Plaut (222) still has value

Oral moniliasis Thrush (Soor, muguet), an infection of the mucous surfaces

of the mouth, is so well known and the causative agent so generally accepted as being *Candida albicans* that there is no need to cite clinical papers. Moniliases connected with other conditions of the oral cavity are not so well known. Reviews of some of the literature on moniliases of the mouth will be found in the papers of Downing (79), Fisher (85), Knighton (146), and Kennedy and Howles (137). Although it is usually admitted that thrush is specifically caused by *C. albicans*, the laboratory diagnosis is not always easy to make, and the same is true for other moniliases of the mouth. It is easy to demonstrate yeast-like cells from the cheek, tongue, lips, teeth, etc., to isolate them and grow them on Sabouraud agar as yeasts, or even to identify them as *C. albicans* in corn meal agar or other appropriate medium. The diagnosis of moniliasis is complicated by the fact that *C. albicans* is present in a number of apparently healthy mouths or may be present possibly as a secondary invader in other pathological conditions.

Many authors have noted the presence of yeastlike organisms in the normal mouth or in mouths free from apparent moniliasis. De Stoecklin (262) found yeastlike fungi in 6% of 330 diphtheritic angina cases and in 16% of 170 non-diphtheritic cases. Tanner, Lampert and Lampert (274) found yeasts including Candida in the mouths of 10% of a large series of healthy young adults. Epstein (79) found the "Soorpilz" in the mouths of 54% of a large series of infants (2 to 6 weeks old), in 46% of those up to 1 year of age, and in 39% of those of the 1 to 6 year age group. The above did not distinguish adequately *C. albicans* from other species. Todd (279) found *C. albicans* in the mouths of 10% of 1002 healthy college students. Fisher (85) found that of 48 children the throats of 12 and the tongues of 6 yielded *C. albicans* and 30 of 122 samples of saliva yielded this species. Knighton (146) isolated yeasts (Candida, Crytococcus and Saccharomyces) from 32% of 123 mouths without dentures and from 61% of 23 cases that were either fully edentulous or with partial plates. However the incidence of *C. albicans* was essentially the same for both series: 24% for the dentate and 22% for the edentulous series. *C. albicans* was considered to be a constant inhabitant in only a portion of the positive cases.

Evidence has been presented (11, 32) that some denture sore mouths are due to *C. albicans*, and there is some evidence (12) for this species being involved in infections of root canals. As indicated by Knighton, (159) more of such evidence is needed to prove that this organism has anything to do with the production of the conditions. Likewise, there is little evidence that Candida is a primary agent in the production of cavities in teeth. Rosebury (241, 242) found "yeast-like organisms" at least 10 times as frequently in mouths containing carious teeth as in those free of cavities. It has been suggested (87) that yeasts (including, no doubt, Candida) in their rapid formation of pyruvic acid from sugars accelerate lactic acid fermentation by oral lactobacilli and thus indirectly promote caries. Infections by Candida following extraction of teeth have been described (106).

Perlèche (83, 89, 212, 239, 254) is an infection of the commissures of the mouth. It is apparently fairly common for in 1929 Finnerud (83) was able to report on 100 cases. The experimental disease was easily produced in human volunteers by pure cultures of *Candida* and *Cryptococcus*. "Monilia" or "Endomyces" have been found (89) in 17% of normal commissures. Isolated strains were

used to produce the experimental disease Many recent writers appear to assume that perlèche is in many cases definitely due to *C albicans*, although it is evident that similar pathological conditions are often due to vitamin deficiencies and that perlèche is often, probably usually, an infection towards which such a deficiency predisposes It has recently been intimated (22) that the connection between perlèche and fungi has been disproved This point of view is evidently not accepted by all pathologists The fact that *C albicans* is found so frequently in the mouths of individuals with no indication of thrush or other oral disease makes it necessary to make diagnoses from laboratory findings with some caution (56) Unless the clinical picture is clear and the organisms are found in abundance, the presence of *C albicans* in the mouth should have no more significance than the presence of *Staphylococcus aureus* in an open lesion Both are pathogens, but both are also normal inhabitants of the body Although it is evident that thrush and other oral moniliases are communicable, it is also evident that some cases may have an endogenous origin The frequent statement that thrush affects largely those whose nutrition is inadequate or unbalanced is based on many observations but can hardly be said to be definitely established, although it is most probably true

Bronchopulmonary moniliasis Bronchomoniliasis was established as a clinical entity by Castellani (35) in 1910 However, as late as 1933, Haler (102, 103) denied that Candida infections of the respiratory tract had been proved He had frequently found Candida present in such infections, but believed that it might well have been a secondary invader Experiments (247) have shown that, unlike the pyogenic gram positive cocci, Candida inoculated together with tubercle bacilli into experimental animals had no effect on the course of the tuberculosis which developed On the other hand, a study (126) of several cases of bronchopulmonary infections suggested that obscure cases not due to other pathogens were frequently Candida infections

In the past 16 years a number of cases of Candida infections of the respiratory tract have been reported (9, 14, 38, 53, 102, 103, 126, 127, 157, 246, 263, 266, 277, 286) Considering these cases in the aggregate (many more might be cited), even if they have not all been adequately proved to be moniliasis, it can hardly be insisted that moniliasis of the lungs, the bronchi, and the larynx does not exist One very recent case report (118), which will be discussed later, is especially convincing Bakst, Hazard and Foley (9) provide a bibliography of cases previous to 1934

The problem of making a diagnosis of moniliasis of the respiratory tract is even more difficult than that of making a diagnosis of oral moniliasis Everyone who has had any experience with the Ziehl-Neelsen staining of sputum must have noted the frequent occurrence of yeast cells, but unless these were isolated and cultured on starvation media, he might not be aware that most of them were species of *Candida* Moreover, as will be shown, they are largely *C albicans* In some cases they may have originated in the mouth, but it is evident that usually they must have come from the lungs or the bronchi It is evident that they are often secondary invaders Emmons (253) has noted that they multiply rapidly after the sample has been taken from the patient A diagnosis

of primary moniliasis of the respiratory tract is to be made only when all other diseases have been ruled out Mendelson (181) in studying sputums from 100 tuberculous suspects found what was considered evidence that 20 of these did not have tuberculosis but moniliasis instead This is a high incidence and it emphasizes the importance of moniliasis in the tuberculosis clinic (14, 37, 118, 121, 122, 184, 224, 287) Moniliasis of the mouth, spreading to the larynx, lips, eyes, and meninges has also been misdiagnosed as non-pulmonary tuberculosis

Table 1 shows the incidence of Candida found in samples of sputum by various investigators

Schwarting chose 67 from the 99 cultures of *Candida* which she obtained from 500 patients (see data in table 1), and found that 58 of these were *C albicans* Furthermore, she found that there was no evidence whatever that Candida tended to be more prevalent

TABLE 1

INVESTIGATOR	KIND OF SPUTUM SAMPLE	NUMBER EXAMINED	PERCENTAGE OF SPECIMENS WITH CANDIDA (ALL SPECIES)	PERCENTAGE OF SPECIMENS WITH C ALBICANS
Jones (130)	Various respiratory conditions	25	44	
Marett (175)	Tuberculosis, known or suspected	568	52	
Marett (176)	" "	2000	75	
Norris (211)	" "	210	8 5	
Maher (174)	" "		10	
Fisher and Arnold (84)	" "	160	28	13
Weedon, Kenney and Shirk (287)	" "	55		9
Burt and Ketchum (31)	" "	693	42	36
Schwarting (248)	" "	500	20	17*

* Estimated

in advanced than in moderately advanced tuberculosis Likewise, there was no evidence of any relationship between cavitation and the presence of Candida in the sputum

The differences in the results in the tabulation above may be more apparent than real It is worthy of note that all who have searched for *Candida* species in sputum samples have found them Moreover, those who have distinguished species have found *C albicans* predominating The fact that such different percentages of sputum samples (some of them possibly aged) were found to be positive can probably be explained by differences in techniques It will make considerable difference whether a loopful or a milliliter sample is taken, whether Sabouraud (pH 5 5) or an acidified medium (pH 4 0) is used and whether fresh or older samples of sputum are examined Unless *C albicans* is found in abundance in fresh sputum, it probably has no diagnostic significance

Vaginal moniliasis Monilia vaginitis and vulvo-vaginitis have been reviewed by Plass, Hesseltine, and Borts (221) As early as 1840, Wilkinson reported

the presence of yeastlike organisms in vaginal discharge, and by 1875, Hausmann was able to report a considerable amount of data which had accumulated in the literature on the subject However, gynecologists of the early years of this century either doubted that the vaginitis had any connection with fungi, or they believed that mycotic vaginitis was very rare It is largely due to the researches of Plass, Hesseltine, and associates (34, 112-117, 220, 221, 298, 300) that a new appreciation of Candida as an important cause of vaginitis is again current Candida is especially likely to occur as a normal parasite in the vagina of diabetics and infections are more likely to occur among them (112-114) Indeed, a common term for the disease has been diabetic vaginitis The reason for this higher incidence is the recurrent washing of skin or mucous surfaces with glucose-containing urine With diet or insulin therapy the condition is usually cured or ameliorated immediately after the disappearance of the glycosuria The disease is much less common in the pre-adolescent and post-menopause years Experimental vulvitis has been produced (117) by applying glucose in powder or solution to normal vaginal surfaces which harbored Candida If yeasts were absent, on the other hand, symptoms were not produced Hesseltine emphasizes that it is not the glucose, acetone bodies or other constituents of diabetic urine which directly cause the symptoms, rather the glucose favors the development of the causative organism already present in the vagina

Candida is more likely to occur in the vaginas of pregnant, than of non-pregnant women, especially during the last three months of pregnancy The increase in the glycogen in the vaginal mucosa during pregnancy is the probable explanation Relief of symptoms or spontaneous cure is usual after delivery The incidence of yeasts in the vaginas of pregnant women is high Woodruff and Hesseltine (300) found 28% of 300 women from all economic classes harbored yeasts in the vagina during the third trimester of pregnancy and nearly half of those harboring yeasts had symptoms of vaginitis These figures include all yeasts and yeastlike fungi and were obtained by examination of smear slides In another series of 152 non indigent patients, 5% had positive slides for yeasts and symptoms of vaginitis and 9 2% positive slides and no symptoms Carter and associates (34) found 43% of 200 pregnant women to harbor yeasts or Candida in their vaginas

Unfortunately, these extensive data do not distinguish the pathogenic *C albicans* from other yeasts Negroni (198) found 8% of 100 non pregnant, as opposed to 33% of pregnant women (196), harbored *C albicans* in the vagina Negroni believed that the habitual use antiseptics or basic washes by 50% of his non pregnant series is responsible for the decreased frequency in this series This is probably a partial but hardly the whole explanation Even if all 8 cultures were obtained from the 50 women who did not admit using these washes, the incidence would still be 16% among the non pregnant women, an appreciably lower incidence than the 33% among pregnant women Thus Negroni's figures become almost exactly like those of Plass *et al* (221) who found 33% of 46 pregnant women and 15% of 39 non pregnant women, all without symptoms of vaginitis, to harbor yeasts in the vagina These last workers did not distinguish species Considerably lower, but showing the same tendency are the figures of Fisher and Arnold (84) who found 15% of 73 women in a prenatal clinic and 6% of 195 in a gynecological clinic had *C albicans* in the vagina In contrast to all these, Castellani and Taylor (42) did not find Candida in the vagina unless there was a clinical disease

The presence of *C albicans* in the vagina, especially if accompanied by vulvitis is said to be an important source of thrush infection for the newborn In 1875 Hausman demonstrated cells of Candida or similar organism in the mouths of newborn from mothers with

vaginal moniliasis Many obstetricians and gynecologists have suggested this as an important source of thrush A paper of Guilini (99) in 1891 was followed by others up to about 1930, when Hesseltine made his more complete study

Candida albicans isolated from the vagina of pregnant women has been used to produce clinical thrush when experimentally inoculated into the mouths of infants (24, 116) This disease was then promptly cured by medication against thrush *Candida Krusei* gave entirely negative results, but 2 out of 12 babies came down with thrush after inoculation with 12 isolates of unclassified Candidas Since the great morphologic variability of *C albicans* was not appreciated when this work was done, these two isolates may well have been *C albicans* It is believed that the mother is an important source of infantile thrush and it has been estimated (300) that the child has 35 times the chance of getting thrush if the mother harbors Candida The incidence of thrush in the Chicago Lying-In Hospital (private) was about 1% It has been recommended (5) that mothers harboring Candida be temporarily separated from their babies It has also been recommended that the pregnant woman be examined in the antepartum period for *Candida albicans* and that it be eliminated if present, by suitable treatment before the child is born Vayssière (282) and Ludlam and Henderson (162) found no connection between vaginal moniliasis of the mother and infantile thrush Another rare infection due to Candida originating from the vagina has been described, namely, infection of the penis of husbands whose wives had vaginal moniliasis Pisacane and Copollino (219) have cited many of the cases of mycotic urethritis of the male reported up to 1938

The work of Jones and Martin (131) on the distribution of species of *Candida* in the vagina has not been mentioned in the above discussion They found yeastlike fungi in the vaginas of 32% of their series of pregnant and 14% of non-pregnant women They also found *C albicans* in the vaginas of women who had clinical vaginitis but not in those of women without symptoms In the normal vagina, however, they found the closely related non-pathogenic *C albicans* var *stellatoidea* (*Monilia stellatoidea*) There seems to be a tendency to ignore this work, but the writer knows of no failure to confirm it If the work cannot be confirmed, this should be put on record If the findings are confirmed, however, the above discussions on vaginal moniliasis and the occurrence of *C albicans* in the normal vagina take on a new meaning of considerably lessened import The ordinary methods of diagnosing *C albicans* such as chlamydospore formation and fermentation reactions, do not distinguish the variety from the species Until it is definitely shown that Jones and Martin were in error in their observation, and as far as the author knows there is no reason to believe that they were in error, the strong probability remains that *C albicans* as distinguished from *C albicans* var *stellatoidea* is not a part of the normal flora of the vagina The source of the pathogen for vaginal moniliasis then, as implied by Mackinnon (168) can best be explained by fecal material which definitely contains *C albicans*

Cutaneous moniliasis This has been the subject of an enormous number of papers in the past twenty years Many of them are of little microbiological interest, rather they belong to the field of dermatology or pharmacology Even eliminating most of these we shall take space to review only a few of the papers cited (10, 15, 33, 65, 123, 125, 172, 186, 193, 194, 251)

The problem of cutaneous moniliasis in some ways differs from that of the oral, vaginal, or bronchopulmonary types of the disease In the first place *Candida albicans* is not found ordinarily on the undiseased skin although other species of the genus are frequently encountered Benham and Hopkins (19), Mackinnon (168), and Croft and Black (62) conspicuously failed to find *C albicans* on the undiseased skin Downing and associates (74) state that they also failed to find this organism on normal skin Conant and associates (56) imply that *C*

albicans is found on the undiseased skin but they cite no evidence Very recently Drake (75) isolated a number of cultures from between the toes of a number of individuals, and accurately diagnosed them as *C albicans* In a personal communication Drake states that in some cases there was undoubtedly intertrigo but in other cases it was doubtful

Secondly, many cases of intertrigo, that is infection of moist surfaces between the toes, fingers or buttocks, or in the inframammary, crural, or axillary regions, and of onychia or paronychia, that is infections of or around the nails, are due to fungi other than *Candida* The dermatophytes are very often the causative agents, and it is possible that other genera of fungi may cause similar diseases, for instance *Cryptococcus* (a number of investigators), *Aspergillus* and *Scopulariopsis* (Negroni, 206) and *Geotrichum* (Cochet, 54) As has been stated (25), the problem of proving the causative agent of cutaneous moniliasis is very like that of proving staphylococcic infections Both organisms are normally present and they can well be and probably often are, mere secondary invaders or chance contaminants This is probably not so with *Candida albicans* since this species is not normally present on the skin It has been suggested (191) that Candida in skin infections is a secondary invader and that this yeast gives rise to symptoms due to allergy See later discussions of sensitivity reactions Mackinnon has stressed the importance of conservatism in attributing this or that organism as the causative agent of a skin infection

Moniliasis of the smooth skin in the moist areas (intertrigo) is not often a serious disease, but it may be somewhat painful and very annoying In one form, Australian surfer's itch, (143) apparently similar to our athlete's foot, it is malodorous Cutaneous moniliasis especially intertrigo is more likely to occur among diabetics (98) and obese individuals due to the increased blood sugar and moist surfaces It has been found (59) that the sweat of individuals with cutaneous moniliasis contains more than the normal amount of sugar It is common knowledge that individuals whose hands are continually wet, as housewives, bartenders and poultry butchers, are more likely to get skin infections on the hands than other individuals This has been established notably by Hopkins and Benham (125) who incidentally were able to reproduce the disease by inoculation of human volunteers with pure cultures In cases of tinea pedis (athlete's foot) or tinea cruris (ringworm of the crotch) or other intertrigous infections, it is often possible to distinguish infections due to Candida from those due to the usual Trichophyton or Epidermophyton by the moistness and redness Cultivation of the organism however is the safest way to actually prove the causative agent

Onychia and paronychia (10, 58, 70, 78, 164, 186, 204, 206, 297) are due to *C albicans* as well as to the dermatophytes and possibly to other species of *Candida* and other fungi These diseases have had considerable study in recent years, especially in Latin America Outbreaks among workers in canneries and in the fruit industry in western United States have been shown to be due to a species of *Candida*, probably *C albicans* (144, 269, 278)

A skin disease, psoriasis, has been connected with Candida Some years ago

(285) it was stated that yeasts and yeastlike fungi were much more numerous in the feces of individuals with psoriasis than in normals, and that these organisms were isolated from the blood stream of a few cases. These observations have not been confirmed and few dermatologists nowadays would connect in any way psoriasis with Candida. Moniliids, often considered with cutaneous moniliasis will be discussed later with immunological reactions of Candida. Loewenthal (161) has reviewed the cases in the literature of skin infections, in the negro, due to fungi. The review is good but the nomenclature of the organisms is not easy to follow.

Intestinal moniliasis. Species of *Candida* have repeatedly been found in fecal material, and they have been suspected but not definitely proved to be the causative agents of several intestinal conditions. Negroni and Fischer (207) give a historical survey up to 1933. Langenbeck, as early as 1839, found yeasts and yeastlike fungi in feces and suggested the connection of these to disease. Casagrandi, in 1898, concluded after considerable work, that yeasts had no connection with diarrhea. It was in connection with tropical sprue that yeasts and yeastlike fungi have been most extensively studied. Kohlbrügge, in 1901, found these organisms in the gastrointestinal tract of well and diseased individuals but much more abundantly in patients suffering from sprue.

Ashford (6–8) found *Monilia psilosis* in considerable number in cases of tropical sprue. This organism was believed to be a separate species on the basis of Castellani's methods, but later it has come to be generally regarded as identical with *Candida albicans*. Ashford studied a very large number of individuals. In one series he found "*M. psilosis*" in the stools of 55% of 280 sprue patients, in 7% of 288 patients with nutritional imbalance, 5% of 126 other patients, and 6% of 178 healthy controls. In a series of 178 healthy boys, 10 had *C. albicans* in their feces, 18 had other species of *Candida*, 51 had other fungi than *Candida* and 99 had no fungi at all. Ashford's earlier conclusion that sprue is due to *C. albicans* (*Monilia psilosis*) was later modified to the point of view that sprue is a nutritional deficiency on which is superimposed a Candida infection (7). To support these views one can also cite Ashford's (6) experiments in which he produced a stomatitis and diarrhea by feeding cultures, and Wood's (299) production of hemolytic anemia in guinea pigs by feeding cultures of the organisms. Anderson (4) also found yeastlike fungi, mostly "wild yeasts" apparently Cryptococcus but also apparently some Candidas in the human intestinal tract, very many more in sprue cases than in patients with other gastro-intestinal disorders or in healthy individuals. Negative results were obtained by feeding these yeasts. Skin tests have been used (142) to indicate that sprue is directly or indirectly a Candida infection. Fisher and Arnold (84) found *C. albicans* in 34% of 69 pathological gastric contents and in 18% of 17 normals. Swartz and Jankelson (270) found the presence of *C. albicans* in stools of non specific ulcerative colitis to be a prognostic sign denoting a malignant course and a possible fatal termination of the disease. The organism was accurately diagnosed by the chlamydospores. Kesten and Suárez (142) found some immunological difference between the *Monilia psilosis* of sprue and the *M. albicans* of intertrigo.

Many recent workers have found no evidence that sprue is in any way connected with yeasts or yeastlike fungi. Mackie and Chitre (163), for instance, found approximately the same number of *Candida albicans* in sprue and non-sprue cases. Many observers (e.g., 19, 86, 105, 153, 207, 213, 247) have found species of *Candida* in feces, often as commonly from healthy persons as from those suffering from sprue or other intestinal disorders. Table 2 will show the incidence of species found by the four of the above who made a careful

differentiation of species In one case species diagnosed as *Monilia stellatoidea* have been listed here as *Candida albicans*, which these authors did not find While it is probable that these species (or varieties) are distinct, it is clear that most investigators have not distinguished between them

It will be noted that all observers have found about the same incidence of *C albicans* in "normal" feces This is remarkable since the observers were widely separated, and the samples were probably obtained from persons of widely different occupation Two of the investigators worked in large cosmopolitan cities, New York and Buenos Aires, and two in smaller centers in largely rural states, Georgia, Oklahoma and North Dakota Their results in this regard check very well with the findings of Nye and collaborators (213), who found that 15 per cent of 192 fecal samples from 121 persons and 31 gastric contents from 29 persons, yielded *C albicans* Felsenfeld (82) also found *C albicans* in feces of 19% of 300 new admissions in a hospital, 38% of 600 ward cases, 34% of 100 typhoid carriers, 41% of 100 "food poisoning carriers", 47% of 47 bacillary dysentery cases and 12% of 50 treated dysentery cases Other species of *Candida*, *Geotrichum* and *Cryptococcus* were

TABLE 2

Incidence of species of Candida in "normal" feces

	REFERENCE			
	(19)	(247)	(207)	(153)
	Feces examined, total			
	100 normal	314 normal	50 surgery cases	102 normal
C albicans	18%	17%	14%	15%
C tropicalis		4%	6%	5%
C Krusei	13%	6%	30%	10%
C parakrusei or *C parapsilosis*	2%	6%	6%	4%
Candida (other spp)	3%		12%	5%

also recorded Fisher and Arnold (84) did not find so many *C albicans*, only 2 out of 39, but 6 *C Krusei* and 3 *Geotrichum* (*Mycoderma*) Most workers have made no distinction between fecal samples with only a few yeasts and those with an abundance of them Schnoor is a notable exception

We must conclude that *C albicans* is a normal parasite in the human intestinal tract, being found in about 15% of normal individuals The evidence for a connection between intestinal disease and Candida is not conclusive and is counterbalanced by considerable negative evidence It has been suggested that the intestinal tract is the great reservoir of *C albicans* (168), and this may well explain the origin of the infection in other parts of the body Castellani (41) on the other hand, attributes the greater incidence of pruritis ani among females than among males to the fact that the organisms are so frequently found in the vagina This opinion, however, would seem to be opposed to Castellani's own findings (42) that Candida was absent in the normal vagina

Generalized moniliasis Moniliasis is to be considered a "superficial mycosis" The organism seems to have an affinity for the mucous membrane and moist skin surfaces and most infections are confined to these superficial areas In onychia and paronychia and in bronchial moniliasis, the infections are somewhat

deeper Six cases of monilial endocarditis have been mentioned in the discussion of *C parakrusei* There are at least three cases of monilial meningitis (104, 184, 255) on record in which Candida was apparently the causative agent, one of these certainly, another probably and the third possibly was *C albicans* From an as yet unreported case of a brain infection (in which *Cryptococcus neoformans* was suspected on account of the numerous yeast cells shown in the *post mortem* sections), numerous colonies were isolated which the writer identified as *Candida albicans* A species of *Candida* was possibly the causative agent in a case of osteomyelitis (57) Besides those just mentioned, clinical papers may be cited (10, 33, 65, 73, 80, 91, 157, 289), in which there was more or less of a generalization of the infection Plaut and Grütz (222) found about a dozen cases in the earlier literature There are probably other papers in the recent literature It is very probable that some of these supposed generalizations of moniliasis were moniliid reactions (see later section) It is evident that Candida does not tend to generalize or to be carried by the blood stream, to secondary foci of infection Such infections occur but they are rare

Moniliasis in animals Little is known as to the distribution of Candida in animals It has been recorded in laboratory animals, rats, hens, rabbits, pigeons and the European hedgehog (60, 66, 273) As a pathogenic organism of turkeys and chickens, *C albicans* is known to cause considerable losses (120, 134, 135) The infection is a thrush-like one and involves the mouth, crop, proventriculus and gizzard In young birds the infection is often fatal, in older ones recovery is usual As to infections of domestic mammals, the writer has found no recent original data Perhaps a quotation from Hagan's (101) text of 1943 is appropriate "Some authors refer to monilia infections of the oral mucosa in calves and colts No additional information about them is available Presumably they are of little consequence " Plaut (222) also found little evidence from the early literature that moniliasis was very prevalent among animals

Experimental pathology of moniliasis The gross or microscopic pathology of moniliasis is not a part of this review Current books on pathology or medical mycology (56, 155) treat this subject Croizat and Rousset (63) discuss the influence of strain differences, age of culture, etc , on the pathology in animals, and Mackinnon (165) discusses the experimental pathology of a number of species of the "genera" of Langeron and Talice Peruchina and Pedace (218) report on the microscopic pathology of experimental moniliasis Mackinnon's (168) summary of the experimental pathology of the various species of Candida is well worth while He shows that *C albicans* caused definite and fatal infections in various laboratory animals, but that of the other species, only *C tropicalis* produced lesions, which were very minor, and rarely fatal Moore (187) has studied the pathology of *C albicans* in the chick embryo

IMMUNOLOGICAL REACTIONS IN MONILIASIS

Antibodies are readily produced by *Candida* species Agglutinin, agglutinin absorption, precipitin and complement fixation reactions (2, 18, 119, 149, 177, 264, 288) can be demonstrated without difficulty As was stated earlier, these

have not been of very great aid in classification since cross reactions between definitely different species are so common Moreover, immune bodies are often found in the serum of persons who have had no history of ever having had monil iasis For these reasons these reactions are of little value in diagnosis of the disease

Recently Drake (75), in a study of the naturally occurring agglutinins for *C albicans* and other yeasts, found that over 90% of the serums of young individuals agglutinated *C albicans* at low titers and 28% agglutinated this organism to as high a titer as certain common yeasts or yeastlike fungi He presented evidence that the incidence is unrelated to the likelihood of exposure to the organism and postulated that these are naturally occurring rather than provoked antibodies In a recent paper (93) unfortunately not available to the writer, it has been reported that the serum of a very small proportion of individuals (only 2 in 1002) gave agglutination reactions with *C albicans* at 1 20 dilution or greater These latter results are not in accord with the findings of most workers

Several writers (139–141, 195, 199, 200, 202, 203, 280, 301)have shown that *Candida albicans* possesses a polysaccharide capsule By separating this polysaccharide from the cells, Negroni was able to show that precipitin and complement fixation antigens are contained in the polysaccharide capsule but that agglutinogens are in the cells proper

The above immunological reactions seem to have little effect upon the course of the disease As in other mycoses, tuberculosis, etc , there is little evidence that these antibodies act against the organisms *in vivo* (124) However, one aspect of the immunological reactions is of utmost importance, sensitization It is evident that most individuals who suffer from moniliasis show a hypersensitivity to Candida, often to a pronounced degree

It is well known that most moniliasis patients are capable of giving a strong skin test if "vaccines" or extracts made from *Candida albicans* are injected intradermally This reactions parallels the tuberculin, the mallein, the trichophytin, and similar reactions and is sometimes called the "oidomycetin" reaction, but the term monilin is probably more common Patients with moniliasis are sensitized to substances present in *C albicans*, and Negroni (202) has clearly shown that these substances are polysaccharide in nature and found in the capsular material This has been confirmed (280) Animal experimentation has also demonstrated that vaccination (injection of killed cells) nearly always sensitizes As with the tubercle bacillus a high degree of sensitivity may be obtained only by actual invasion and survival of the living cells in the tissues With Candida, however, a weaker sensitization may be obtained by repeated injections of dead cells or their products It has been shown (205, 268) that tissues can be passively sensitized by a technique similar to that used in the Prausnitz-Kuessner reaction Since *Candida* species are found so frequently in the normal body, very often persons with no history of moniliasis may give a stronger or weaker skin test It is to be expected that a large number of individuals should thus become sensitized (56, 138, 142, 155, 259), hence the skin test has limited diagnostic value Some (e g , 156) for instance, do not recommend it at all Others (138) recom-

mend it in conjunction with a similar trichophytin test for cutaneous infections but minimize the importance of a positive monilin reaction. It has been recommended (56) that, inasmuch as KI therapy is dangerous to strongly sensitized persons, the skin test should always be given, not for diagnosis but to determine if desensitization is indicated. Kerr, Pascher, and Sulzberger (138) give a good review of monilin skin tests.

In some mycoses it is probable that sensitization occurs before a permanent infection takes place (110), that is, "sensitization is prerequisite to infection". In Candida infection this is not necessarily true, but animal inoculation has shown that intradermal injections of the organism into sensitized animals give a more acute inflammatory reaction than do inoculations into non-sensitized animals (155, 172, 214). This is comparable to the Koch phenomenon.

The sensitivity is not entirely specific. Owen, Anderson, and Henrici (214) show that within the genera *Candida* and *Cryptococcus* there are many cross reactions. However, cross reactions with sporotrichin, tuberculin, and trichophytin are absent, or at low titer. The ability of strains to sensitize is correlated with their potential virulence. Thus the above workers found that isolates of *Candida* pathogenic for one species of laboratory animal produced more sensitivity and stronger reactions in the same species of animal than did nonpathogenic isolates. Thermostable substances in the cells of *C. tropicalis* sensitize guinea pigs to anaphylaxis (158).

Related to the skin test is the allergic id (moniliid) reaction (92, 110, 125, 155, 214). This is manifested by the appearance of a secondary scaly erythematous inflammation on the skin far removed from the site of infection. It does not appear in all cases of moniliasis and never in an individual who does not give a strong skin test. Similar id reactions are more or less common in other diseases in which sensitivity is a prominent part of the pathology (tuberculid, trichophytid, sporotrichid, etc.). The reaction is apparently the response of the sensitized skin to the allergens brought there by the blood stream. It is possible that in some cases the whole cell is transported, but the lesion is nearly always sterile. The id reaction may be simulated after putting some of the allergen directly into the skin, but in this case we call it a "skin test" reaction.

Moniliids may be severe, chronic, or even fatal. Apparently the first to study them were Revaut and Raveau (235) in 1928. Yeasts were recovered from intertrigous lesions, but the secondary lesions of the arms and legs were free of fungi. All patients gave a positive skin test to extracts of the yeast, and when the primary lesion was cured by medication the secondary lesions disappeared. The authors postulated the theory which is still held, that these secondary lesions were typical id reactions, that is that the sensitized skin reacted to the allergens carried thither by the blood stream. These findings were soon confirmed (92) and the subject has been discussed by Hopkins (125) who has cited many case reports.

In relation to immunity reactions a recent case report (118) is very instructive. A patient with severe pulmonary moniliasis from whose sputum *C. albicans* was repeatedly isolated, had a negative skin test and a negative agglutination

reaction The patient was not sensitive to normal rabbit serum Anti-Candida rabbit serum, however, produced a very severe reaction The explanation for these phenomena was that the "patient was flooded with an excess of the fungus antigen which inhibited both the agglutination reaction and the skin test" After receiving many small but increasingly larger amounts of anti-Candida serum, the patient eventually had a total remission of symptoms, the skin test for Candida became positive and the agglutination titer reached 1 80 A similar failure of agglutination reaction in a very severe case of moniliasis has been recorded (240) The analogous failure to get a positive Mantoux test in the latter stages of tuberculosis is well known

There have been several reviews on sensitivity reactions induced by yeasts and related organisms (23, 44, 110, 133, 214, 231)

BRETTANOMYCES

Morphology The genus *Brettanomyces* is known almost entirely from the dissertation of Custers (64) A copy of a translation by Phaff and Douglas of this important paper was kindly loaned to the author by Mrak Diddens and Lodder (67) at first apparently had not expected to recognize *Brettanomyces* as a genus separate from *Candida*, but finally did so in their monograph (69) There is only one clear-cut morphological distinction between the genera, namely the ogival shape of some of the individual cells in cultures of *Brettanomyces* These pointed cells of Gothic shape are distinct, but they seem hardly sufficiently important for separating genera The other morphological characters given, the poorly developed pseudomycelium and the lack of "blastospore apparatus" can be found also in species of *Candida* (see Diddens and Lodder's (69) drawings) However, since Diddens and Lodder have chosen to recognize the genus *Brettanomyces* we shall do the same

The poorly developed pseudomycelium, the supposedly different kind of blastospore production and the pointed cells do not notably separate *Brettanomyces* from *Candida*, but there are physiological differences which make the former genus of some industrial importance Growth is rather slower than in most yeasts and colonies appear on plates from mixed cultures after most yeast colonies have attained a large size This possibly accounts for the fact that little is known of this genus among most workers in yeasts Undoubtedly they have sometimes been present but missed Brettanomyces can be found in the late fermentations in the manufacture of certain beers, especially in the types known as Belgian lambic and in English porter, pale ale, and stout Here it continues the alcoholic fermentation after the Saccharomyces (usually top yeasts) have ceased action, and as much as 10% alcohol may be produced Lambic beer is lagered for years Under aerobic conditions acid is produced, hence the beer must be kept anaerobic Either calcium carbonate must be used in the agar slant culture media or else the cultures must be incubated anaerobically Otherwise the acid kills the culture

Biochemistry In contrast to that of Candida, the physiology of Brettanomyces has been studied thoroughly Since Custers' thesis is in Dutch and not easily obtainable, space will be taken to give a résumé of its biochemical parts

Ethyl alcohol and carbon dioxide are the only products of the anaerobic dissimilation of glucose by *Brettanomyces Claussenii* Two molecules of ethyl alcohol and two of CO_2 are produced from one molecule of glucose, as in ordinary yeasts In yeast water or yeast autolysate media slightly more than the theoretical amount of alcohol is formed and this extra alcohol with less than the expected CO_2 comes from the organic components of the yeast water or yeast autolysate Only cells which have been previously cultured anaerobically for some time are able to ferment anaerobically with any great facility At best the speed is much slower than that of ordinary fermenting species of *Saccharomyces*

When *B Claussenii* and *B bruxellensis* are cultured aerobically on a glucose-containing substrate, only CO_2, ethyl alcohol, and acetic acid are produced The acetic acid is produced, not from the sugar, but from the alcohol At pH 6 4 only acetic acid is formed from the alcohol, but at pH 4 35 and 3 77 some of the acetic acid is further oxidized to CO_2 In *Saccharomyces cerevisiae* the oxidation of the acetate, which may be formed under some circumstances, is much less dependent on the pH of the medium, and acetate does not accumulate

Of special interest is the fact that *Brettanomyces Claussenii* shows a definite negative Pasteur effect, that is, when a glucose broth culture is inoculated with cells previously grown aerobically, the cells under the more anaerobic conditions at the bottom of the flask are unable to carry on more than a very slight anaerobic fermentation and an aerobic fermentation only in proportion to the amount of dissolved oxygen As this oxygen is used up, the fermentation of these cells decreases At the surface the cells are more able to ferment due to the greater amount of oxygen present, but as the air is exhausted and the medium becomes anaerobic clear to the surface, the fermentation rate decreases Thus we have the peculiar condition of a seeming decrease in both aerobic and anaerobic fermentation at the same time, as air is exhausted Slowly the cells become more and more adapted to anaerobic conditions and the normal anaerobic fermentation becomes established Manometric studies also show that more fermentation takes place aerobically than anaerobically in a given time if cells with a previous history of aerobic culture have been used The fact that for some time aerobic production of alcohol proceeds at a more rapid rate than anaerobic fermentation can be called a negative Pasteur effect However, if cells, previously grown anaerobically are used as an inoculum, the normal Pasteur effect is noted, that is, the anaerobic dissimilation of glucose proceeds more rapidly than the aerobic dissimilation

Custers showed that while other yeasts do not normally show a negative Pasteur effect, there is some indication that the same phenomenon may be demonstrated under special conditions It is common knowledge that many disaccharides will serve as good sources of carbon for certain yeasts which cannot ferment them The usual explanation is that the molecule is attacked in some other way than by hydrolysis into two monosaccharides Custers was able to demonstrate that a certain yeast which was able to utilize maltose aerobically, but which also was totally unable to ferment it anaerobically actually produced maltase aerobically, and under certain conditions could ferment maltose aerobically Thus this yeast which with glucose shows the normal Pasteur effect

(i e, the intensity of the fermentation is greater under anaerobic than under aerobic conditions, regardless of the previous history of the culture), actually could be made to show a negative Pasteur effect with maltose Under aerobic conditions maltose was fermented, but under anaerobic conditions not at all The maltase is completely inhibited in the absence of air A similar phenomenon is produced by certain other yeasts in media containing lactose (145)

Classification Custers described four species of *Brettanomyces*, two of them new, and two new varieties *B bruxellensis*, *B bruxellensis* var *non-membranefaciens*, *B bruxellensis* var *lentus*, *B lambicus*, *B Claussenii*, and *B anomalus* He noted the close relationship of *Brettanomyces* to *Candida intermedia* but decided to keep this latter species in the genus *Candida* Specific distinctions were made partly on a morphological basis, but mostly on the basis of fermentation of sugars, and appearance on agar or liquid media The distinctions between species seem definite enough. All the isolates which Custers studied were of brewery origin Diddens and Lodder made no taxonomic change in Custers' treatment of the genus and they found no habitat not connected with fermentation industries According to Smith (Industrial Mycology, 1946) species of *Brettanomyces* are of fairly common occurrence in English breweries and may constitute a source of trouble in beers which are not sufficiently matured Objectionable odors are apparently produced in the earlier stages of storage

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DYNAMICS AND MECHANISM OF IMMUNITY REACTIONS IN VIVO

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Whenever immune serum is administered for prophylactic or therapeutic purposes, pathogenic agent and antibody meet somewhere in the animal body and the reaction between the two components is appropriately described as an immunity reaction *in vivo*. These reactions are very different from the reactions with which the immunologist is usually concerned. In standardization work especially, the pathogenic agent and the antiserum are mixed *in vitro* before being injected into the experimental animal. The entire reaction takes place in the test tube and the experimental animal serves only as an indicator for the unneutralized pathogenic agent. Despite the use of experimental animals, these reactions are in reality immunity reactions *in vitro*. Thus far, immunology has been essentially a science of immunity reactions *in vitro*. Relatively little work has been done on immunity reactions *in vivo*. The present review is concerned with this particular aspect of immunology. The author and his collaborators have been concerned with this subject for some years. The interest was essentially a practical one. It was hoped that a more intimate knowledge of immunity reactions *in vivo* would furnish, or at least contribute to, a much needed theoretical foundation in serotherapy and seroprophylaxis.

Our experimental procedure was developed from this point of view. In clinical medicine one speaks of serum therapy if the serum is administered at a time when symptoms of systemic disease are already present. It appeared logical to imitate these conditions by producing the disease in the experimental animal and administering the serum as soon as the animal became sick. Experience has shown however, that it is hardly possible to work along these lines under reproducible conditions. Several authors (6, 7, 26, 49) have injected the serum at various intervals after the toxin, but before the appearance of clinical symptoms. This method has led to important results, but it has the disadvantage that nothing is known of the fate of the toxin at the time of the administration of the antitoxin.

The appearance of symptoms of a systemic disease is a certain sign that the pathogenic agent has reached susceptible tissues. As will be shown in the following sections, it is not so much the presence of symptoms as the presence of the pathogenic agent in susceptible tissues that is of paramount importance for the antiserum requirements. To work under well defined experimental ...ditions and to imitate at the same time the therapeutic use of antiserum as ...losely as possible, we injected the pathogenic agent directly into some suscep... ...e tissue (skin, brain, or muscle) while the serum was given intravenously ... a constant dose of the pathogenic agent and serial dilutions of serum were ... The serum was injected immediately before the pathogenic agent. Ex... ...f this type will be designated as *indirect tests* and the minimal neu... ...mounts of immune serum in these tests as *Ai*

The neutralizing dose of antiserum *in vitro* was determined with the aid of the *direct test*. In this test a constant dose of pathogenic agent and serial dilutions of antiserum were mixed and 0.1 ml of each mixture injected into the same tissue as in the indirect test. The minimal neutralizing amount of immune serum in the direct test will be designated as *Ad*.

Actual interest lies in the ratio *Ai/Ad*, i.e., the ratio between the neutralizing dose *in vivo* and the neutralizing dose *in vitro*, and since *Ai* is closely related to the therapeutic dose, one could also say that the ratio is that of the therapeutic dose to the neutralizing dose of antiserum. This ratio is independent of the potency of the immune serum and of the combining power of the test dose of the pathogenic agent. It is exclusively determined by the mechanism of immunity reactions *in vivo*. With the aid of appropriate experiments, therefore, this mechanism can be deduced from the *Ai/Ad* ratio. In the experiments to be discussed this ratio was determined for a variety of pathogenic agents and submitted to further experimental analysis.

TABLE 1

Determination of the Ai, Ad and Ai/Ad values in skin tests with diphtheria toxin

The *Ai* and *Ad* values were determined for 3 different test doses of diphtheria toxin. In three parallel experiments serial dilutions of antitoxin were injected intravenously in white rabbits weighing 2500 g. Immediately afterwards 0.1 ml of diphtheria toxin was injected intracutaneously, in the first series in a dilution 1/10,000, in the second in a dilution 1/2,000 and in the third in a dilution 1/50. The results read after 48 hours were:

TEST DOSE OF TOXIN (0.1 ML)	DOSE OF ANTITOXIN		
	Ai (1 ml)	*Ad* (0.1 ml)	*Ai/Ad*
1/10,000	1/200	1/400,000	20,000
1/2,000	1/50	1/80,000	16,000
1/50	1/1	1/3,000	30,000

The Ai/Ad ratio and the distribution of antibody between blood and tissues

A well known example of a local reaction is the skin test with diphtheria toxin. In table 1 is recorded, in abbreviated form, an experiment in which the *Ai/Ad* ratio was determined (13) for diphtheria toxin in rabbits. Table 1 shows that, within the limits of experimental error, the *Ai/Ad* ratio is independent of the test dose of the toxin and has a value of approximately 22,000 in the rabbit. With certain variations, this figure probably holds true for other tissues, such as the heart and the adrenals. It can be concluded, therefore, that the therapeutic dose of diphtheria antitoxin will be at least 20,000 times higher than the neutralizing dose.

The high value for the *Ai/Ad* ratio becomes understandable when it is considered that only a tiny fraction of the intravenously injected antitoxin reacts with the toxin in the skin. It is very likely, therefore, that the reaction between toxin and antitoxin is determined by the relative concentrations of the two components in the tissue and not by the absolute amounts. The two experiments (13), recorded in tables 2 and 3, show the correctness of this assumption.

It will be seen from table 2 that the endpoint of titration is the same in both series, although 10 times more toxin was employed in the first series (column 2) than in the second (column 3) This experiment shows clearly that the outcome of the skin test is exclusively determined by the concentration of the toxin in the skin and is independent of the absolute amount

TABLE 2

Dependence of the result of the skin test on the concentration of the toxin in the skin

1 ml of 1/50 diphtheria antitoxin was injected intravenously into a white rabbit weighing 2500 g Immediately afterward serial dilutions of diphtheria toxin were injected intracutaneously, in one series in a volume of 0 1 ml and in the other in a volume of 0 01 ml

TOXIN DILUTION	0 1 ML TOXIN RESULTS*	0.01 ML TOXIN RESULTS*
1/100	E++	E++
1/200	E++	E++
1/400	E++	E+
1/800	0	0
1/1600	0	0
1/3200	0	0

* E = erythema, 0 to ++++ indicates degree of erythema

TABLE 3

Dependence of the result of the skin test on the concentration of the antitoxin in the skin

A 1 ml dose of diphtheria antitoxin, diluted 1/20, was injected intravenously into a rabbit weighing 2500 g, and also into a guinea pig weighing 500 g Immediately afterwards skin tests with diphtheria toxin were performed

TOXIN DILUTION (0.1 ML)	OUTCOME IN RABBIT*	OUTCOME IN GUINEA PIG*
1/50,000	0	—
1/20,000	0	—
1/10,000	0	—
1/5,000	0	0
1/2,000	E++++ N+	0
1/1,000	E++++ N+	0
1/500	E++++ N+	N+
1/200	—	N+++
1/100	—	N+++

* E = erythema, N = necrosis, 0 = no reaction, — = not done

Table 3 shows that, for the same dose of antitoxin, the maximal amount of neutralized toxin is inversely proportional to the body weights of the experimental animals This result strongly suggests that the outcome of the skin test depends on the concentration of the antitoxin in the blood plasma and is, therefore, determined by its concentration in the skin

There is little doubt that the high *Ai/Ad* ratio, in the skin test with diphtheria toxin, is due chiefly to the dilution of the antitoxin in the blood plasma and to its low concentration in the skin tissue This also seems to follow from the fact that the same *Ai/Ad* ratio of 22,000 was found (13) in experiments with staphy-

lococcus toxin. It is of great interest to calculate the influence of these two factors on the Ai/Ad ratio. Assuming that other factors can be neglected, the minimal neutralizing concentrations of antitoxin in the skin will be identical in the direct and the indirect tests. This concentration is Ad/V in the direct test and Ai/PK in the indirect test.

$$\text{Hence, } Ai/Ad = PK/V, \text{ or } K = AiV/AdP \qquad (a)$$

In these formulas V represents the volume of intracutaneously injected fluid, P the plasma volume, and K the coefficient of the distribution of antitoxin between plasma and skin. Were the ratio exclusively determined by these factors, it should obviously have the same value for all pathogenic agents. Actually immunity reactions *in vivo* may be and often are of a much more complicated character. It will be seen later that the tissue cells may take part in several ways in the reaction between pathogenic agent and antibody and thereby control the Ai/Ad ratio. In this case equation *a* does not hold true. Nevertheless, the equation is not altogether worthless. It indicates what the Ai/Ad ratio would be if it were exclusively determined by the dilution of the antitoxin in the blood plasma and its low concentration in the skin, irrespective of the question whether this condition is ever realized.

It is a fortunate circumstance that the skin test with diphtheria toxin furnishes the material needed for the application of the equation. It has been shown (12) that the reaction between diphtheria toxin and antitoxin is extremely rapid. In the skin, the reaction is complete before the toxin has opportunity to react extensively with the tissue. Therefore, the velocity of the reaction between toxin and tissue is without importance for the Ai/Ad ratio and equation *a* is valid.[1] This is proved independently by the following consideration. K can be calculated from the equation if Ai and Ad have been determined experimentally. If $Ai/Ad = 22{,}000$, $P = 100$ and $V = 0.1$, then K has the value 22, i.e., the concentration of antitoxin in the skin is approximately 5% of that in the blood plasma. Since antibodies (in this case, diphtheria antibody) are either globulins or closely linked to them, K should have the same value for antitoxins and globulins. This is actually the case. According to Peters (44), the globulin content of the tissue fluid is approximately 5% of that of the blood plasma.[2]

With a view to later experiments with neurotropic viruses, the Ai/Ad ratio was determined for diphtheria toxin in intracerebral tests in guinea pigs weighing 250 g (14). The value found was 6200. If the body weights of the two species (rabbit and guinea pig) are taken into consideration, it is apparent that the

[1] The lengthy but conclusive proof of this statement would interrupt the flow of presentation. The interested reader may be referred, therefore, to the original paper (12).

[2] This result meets a criticism (53) that has been levelled against our method of calculation. It has been claimed that diphtheria toxin is a capillary poison and that, consequently, the method cannot possibly measure the normal permeability of the capillaries. In this case, however, K could not have the same value for antitoxins and globulins. The reason for the validity of the equation, in this case, is the long incubation period of diphtheria toxin. In the skin test it takes many hours before any effect of the toxin on the capillaries becomes visible in the form of erythema. Long before that can happen, the toxin is neutralized by the antitoxin (12).

Ai/Ad ratio for the brain is higher than for the skin. That is as it should be, for according to Freund (9) the antibody concentration in the brain is lower than in most other tissues.

The value 6200 is of great importance. It shows what the *Ai/Ad* ratio would be for neurotropic viruses if it were determined simply by the distribution of the antibody between blood and brain. It will be seen in a subsequent section that the deviation of the experimentally determined *Ai/Ad* ratio from the figure 6200 gives valuable information concerning the mechanism of immunity in neurotropic virus diseases.

This may be the opportunity to mention some earlier investigations although they are only loosely connected with the subject. It was early recognized that the therapeutic dose of antitoxin was infinitely greater than the neutralizing dose and several attempts were made to explain this difference. All these investigations have in common the fact that the antitoxin was injected at various intervals after the toxin.

Doenitz (6, 7) injected 15 MLD of diphtheria toxin intravenously into rabbits, forty minutes later even 11,000 times the amount of antitoxin which neutralized the test dose of toxin *in vitro* was unable to protect the animals. Schick *et al.* (49) determined the amount of intramuscularly injected antitoxin which prevented a positive Schick test in children. The Schick reaction failed to appear when the antitoxin was injected simultaneously with the toxin, but was positive when antitoxin was injected 3 or 6 hours after the toxin.

Glenny and Hopkins (26) injected diphtheria toxin intracutaneously into rabbits and, after various intervals, antitoxin was injected intravenously. Fifteen minutes after the intracutaneous injection of the toxin, 10,000 times the neutralizing dose of antitoxin was unable to prevent a positive skin reaction. After thirty minutes even one million times the neutralizing dose was ineffective.

From these experiments it has been concluded that the combination between toxin and tissue undergoes a gradual increase in firmness and that, consequently, it becomes increasingly difficult for the antitoxin to dislodge the toxin from the cells. I think it is still the general opinion that this reaction between the toxin and the tissue is the only reason for the difference between the neutralizing and the therapeutic dose of antitoxin. The experiments reported in the first part of this section show that this is not the case. The most important factor is the separation of the toxin in the tissues from the antitoxin in the blood by the capillary wall. It is very likely, also, that this factor plays an important role in the interpretation of the experiments of Doenitz and of Glenny and Hopkins. Nevertheless, there is no doubt that the difference between the therapeutic and the neutralizing dose of antitoxin is at least partly due to the reaction between toxin and tissue. The *Ai* value, therefore, differs from the therapeutic dose by a factor which takes account of this reaction.

The systemic action of diphtheria toxin and the Ai/Ad ratio. Pathogenesis of tetanus

In the preceding section the skin test with diphtheria toxin was discussed as an example of the local action of the toxin on the tissue into which it had been

injected In the present section the action of the toxin on organs remote from the site of its injection will be described It will soon be seen that this experiment has some important implications

A representative experiment is recorded in table 4 (21) As may be seen from this table, intramuscularly injected toxin does not require even twice as much antitoxin as intravenously injected toxin The antitoxin requirements would probably be exactly the same were it not for the effect of the Danysz phenomenon The toxin is slowly absorbed from the muscle, and therefore, requires more antitoxin than the same dose of toxin injected intravenously Accordingly we have found in unpublished experiments that intravenously injected toxin required for its neutralization the same amount of antitoxin as intramuscularly injected toxin, if the former was injected in four portions separated from each other by intervals of two hours The explanation of these experiments is simple

TABLE 4

Antitoxin requirements for intramuscularly and intravenously injected diphtheria toxin

Serial dilutions of diphtheria antitoxin were injected intravenously into guinea pigs weighing 250 g Immediately afterward, 20 lethal doses of diphtheria toxin were injected intramuscularly in series A, and intravenously in series B Death or survival of the experimental animals was recorded

ANTITOXIN (0.5 ML)	SERIES A*	SERIES B*
1/100	S,S,S,S	S,S
1/200	S,S,S,S	S,S
1/400	S,5,6,5	S,S
1/800	2,2,2,2	S,1,2,3,5

* S = survived, numerals indicate day of death

In order to protect the animals in these experiments, it is necessary to neutralize the toxin before it reaches the heart or the adrenals, but it is immaterial whether it is neutralized at the site of its injection or in the blood Theoretically, therefore, intravenously and intramuscularly injected toxin should be neutralized by the same amount of antitoxin

It is interesting to compare the result of the indirect intramuscular test with that of the indirect intracutaneous test To do this it will be convenient to define the direct test in a more general way As before, the term "indirect test" will be the designation for experiments in which toxin and antitoxin are injected into separate compartments The term "direct test", however, will now include all experiments in which toxin and antitoxin are injected into the same compartment, e g, the vascular system The minimal neutralizing amount of antitoxin in such an experiment will again be designated as *Ad* The above results can now be formulated as follows In the skin test, in which the local action of the toxin was considered, the *Ai/Ad* ratio was 22,000 In the intramuscular test, in which the systemic action of the toxin was dealt with, the *Ai/Ad* ratio approached unity The reason for this is the fact that in the skin test, in order to obtain the

result, it is necessary that the toxin be neutralized at the site of its injection This, however, is immaterial in the intramuscular test

These rather obvious considerations found an unexpected and important application in the case of tetanus toxin If this toxin reached the central nervous system by way of the circulation, the Ai/Ad ratio in the indirect intramuscular test should be the same as with diphtheria toxin, i e, unity In this case it would be immaterial whether the toxin is neutralized at the site of its injection or in the vascular system If, on the other hand, according to Meyer and Ransom (38) the toxin reaches the central nervous system by way of the peripheral nerves, the animals can be protected only if the toxin is neutralized in the muscle This neutralization, in turn, depends on the concentration of the antitoxin in the muscle tissue Therefore, the Ai value and consequently, the Ai/Ad ratio must be high By determining this ratio it should be possible, therefore, to decide

TABLE 5

Antitoxin requirements of intramuscularly and intravenously injected tetanus toxin

In two series of experiments, serial dilutions of tetanus antitoxin were given intravenously to guinea pigs weighing 250 g Immediately afterwards 20 lethal doses of tetanus toxin (Lederle) were injected intramuscularly in one series and intravenously in the other

ANTITOXIN (0.5 ML)	TOXIN INTRAMUSCULARLY RESULTS*	TOXIN INTRAVENOUSLY, RESULTS*
1/20	L T	—
1/40	L T	—
1/100	5	—
1/200	5	—
1/400	5	—
1/800	3	S
1/1600	—	S
1/3200	—	S
1/6400	—	3

* L T = local tetanus, S = survived, — = not done, numerals indicate day of death

whether tetanus toxin reaches the central nervous system by way of the circulation or by way of the peripheral nerves The experiment recorded in table 5 gives a clearcut answer to this question (16) As may be seen, the Ai/Ad ratio in the indirect intramuscular test as defined above, is 80 1, whereas it was near to unity in the case of diphtheria toxin

Further experiments have shown very clearly that the high Ai/Ad ratio for tetanus toxin can be explained only by the migration of the toxin in the peripheral nerves It was found that after sectioning of the sciatic and femoral nerves the Ai/Ad ratio attained the same value as for diphtheria toxin, namely, unity (17) This experiment is demonstrated in table 6

The following experiments show that in accordance with the theory of Meyer and Ransom only sectioning of the motor nerve roots has this effect, whereas sectioning of the posterior nerve roots has no influence on the Ai/Ad ratio (18) These experiments were carried out in rhesus monkeys In preliminary tests

it was determined how much intravenously injected antitoxin was necessary to protect the monkeys against 25 MLD of tetanus toxin given either by the intramuscular or intravenous route, 1 ml of a 1/3200 dilution of antitoxin protected against the intravenously injected toxin, whereas the intramuscularly injected toxin required 1 ml of a 1/100 dilution In the main experiment, 1 ml of a 1/600 dilution of antitoxin was given The intramuscularly injected control animals died within a few days Four animals in which the posterior nerve roots had been transsected prior to the experiment, died at the same time as the controls Three other monkeys whose anterior nerve roots had been cut survived the injection of a dose of toxin which killed the controls and the animals with the

TABLE 6

Antitoxin requirements for intramuscularly and intravenously injected toxin and for intramuscularly injected toxin after denervation of the leg

Serial dilutions of tetanus antitoxin were given intravenously to guinea pigs weighing 250 g In series I, 20 lethal doses of tetanus toxin (Lilly) were given intramuscularly, in series II, intravenously In series III, the femoral and sciatic nerves were cut before the intramuscular injection of the toxin

ANTITOXIN (1 ML)	SERIES I TOXIN INJECTED INTO INTACT MUSCLE RESULTS*	SERIES II TOXIN INJECTED INTRAVENOUSLY RESULTS*	SERIES III TOXIN INJECTED INTO DENERVATED MUSCLE RESULTS
1/16	L T	S	S
1/128	L T	S	S
1/256	3	S	S
1/512	2	S	S
1/1,024	2	S	S
1/2,048	—	G T	L T
1/4,096	—	2	3
1/8,192	—	2	—
1/16,384	—	1	—

* L T = local tetanus, G T = generalized tetanus, S = survived, — = not done, numerals indicate day of death

dissected posterior nerve roots This experiment shows that the Ai/Ad ratio is high only when the motor nerve pathway is intact

From the observations discussed in this section follow some important general rules concerning the Ai/Ad ratio

1 The Ai/Ad ratio is always high when at the time of injection of the antitoxin the toxin is already in the susceptible tissue (as in the skin test with diphtheria toxin)

2 If at the time of the injection of the antitoxin, the pathogenic agent is still outside the susceptible tissue (indirect intramuscular test with diphtheria toxin) the Ai/Ad ratio approaches unity, provided that the pathogenic agent reaches the susceptible organ by way of the circulation

3 Under the same circumstances the Ai/Ad ratio is high if the pathogenic agent reaches the susceptible organs along the nerve pathways

Quantitative determination of capillary permeability with the aid of the indirect test (22)

In the course of the investigations, it was observed that, in the indirect skin test with diphtheria toxin, the effect of antitoxin was strongly enhanced when the toxin was dissolved in the solution of certain substances other than NaCl This effect was very marked with nutrient broth as may be seen from table 7 Twenty-two times more toxin was neutralized in series B (solvent, broth) than in series A (saline) The result of the direct skin test was in no way affected by broth, hence the possibility that broth has a direct influence on the reaction between toxin and antitoxin was excluded

TABLE 7

Antitoxin requirements in indirect skin tests with diphtheria toxin dissolved in saline or nutrient broth

1 ml of 1/50 diphtheria antitoxin 874 (1600 units per ml) was injected into a white rabbit weighing 2500 g Immediately afterwards serial dilutions of diphtheria toxin 1116 in a volume of 0 1 ml were injected intracutaneously In series A, the toxin was diluted in saline, in series B, it was diluted in nutrient broth Results were read after 48 hours

TOXIN DILUTIONS	SERIES A RESULTS*	SERIES B RESULTS*
1/100	E	N
1/150	—	N
1/200	E	E
1/250	—	E
1/300	—	0
1/400	E	0
1/800	E	0
1/1600	E	0
1/3000	E	—
1/4500	E	—
1/6000	E	—
1/6500	0	—
1/7000	0	—

* N = necrosis, E = erythema, 0 = no reaction, — = not done

The result would obviously be explained if it were assumed that broth increases the permeability of the capillaries to antitoxin There were several ways whereby the correctness of this assumption could be verified The increased permeability to antitoxin should influence the indirect test only when the concentration of the antitoxin at the site of injection of the toxin determines the result of this test According to the findings in the preceding section, therefore, broth should increase the effect of antitoxin in the intramuscular test with tetanus toxin but should fail to do so in the intramuscular test with diphtheria toxin Tables 8 and 9 show that this is exactly what happened

As may be seen from table 8, the antitoxin titer was the same in both series In other words, broth had not altered the result of the indirect intramuscular

test with diphtheria toxin As table 9 indicates, the animals in series B (broth) were protected by ⅛ of the amount of tetanal antitoxin which was required in series A (saline) These experiments show clearly that broth enhances the effect of antitoxin in the indirect test with tetanus toxin, by increasing the permeability of the capillaries to the antitoxin

TABLE 8

Indirect intramuscular test with diphtheria toxin dissolved in saline or nutrient broth

Serial dilutions of diphtheria antitoxin 874 (1600 units per ml) were injected intravenously in 1 ml amounts into guinea pigs weighing 250 g Immediately afterwards, 20 lethal doses of diphtheria toxin 1116 were injected intramuscularly In series A, the dilutions of toxin were made in saline, in series B, they were made in broth

ANTITOXIN (1 ML)	SERIES A (SALINE) RESULTS*	SERIES B (BROTH) RESULTS*
1/200	S,S	S,S
1/400	5,6	3,3
1/800	2,2	2,2

* S = survived, numerals indicate day of death

TABLE 9

Indirect intramuscular test with tetanus toxin dissolved in saline or nutrient broth

1 ml amounts of serial dilutions of tetanus antitoxin 387 (1200 units per ml) were injected intravenously into guinea pigs weighing 250 g Immediately afterwards, 20 lethal doses of tetanal toxin 1556 in a volume of 0 1 ml were injected intramuscularly In series A the toxin was diluted in saline, in series B, in broth

ANTITOXIN (1 ML)	SERIES A (SALINE) RESULTS*	SERIES B (BROTH) RESULTS*
1/10	L T , L T	—
1/20	L T , L T	—
1/40	6,6	S,S
1/80	2,2	L T , L T
1/160	3,3	L T , L T
1/320	—	L T , 5
1/640	—	2,2
1/1280	—	—

* L T = local tetanus, S = survived, — = not done, numerals indicate day of death

The effect of broth on the capillaries could be demonstrated even more directly with the aid of aniline dyes Five ml of a 1% solution of Evans blue was injected into a white rabbit weighing 2500 g The depilated skin assumed a very slight blue color The intracutaneous injection of 0 1 ml of saline produced no visible change in the skin After the injection of 0 1 ml of broth even in a dilution of 1/30, however, the bleb stained a deep blue

The indirect intracutaneous test with diphtheria toxin and the indirect intramuscular test with tetanus toxin have been employed in a study of the effect of a number of chemicals and biological fluids on the permeability of the capil-

laries The results are briefly summarized in table 10 Although the effect of substances on capillary permeability can be and has been studied with the aid of physiological methods, the latter are mostly of an indirect nature and measure the effect on lymph production rather than on capillary permeability directly Both do not necessarily run parallel Egg albumin, for instance, is a strong lymphagogue, but in our experiments it had no effect on the capillaries of the skin Duran-Reynals (8) found that testicular extract dilated the capillaries but left unanswered the question whether or not this effect was due to the spreading factor Our quantitative method permitted the demonstration that the spreading factor is destroyed by boiling for a few minutes while the capillary factor in testicular extract is thermostable

Of particular interest is the effect of the blood group substances on capillary permeability These substances were investigated because they are present in commercial peptones The substances with blood group properties, isolated by

TABLE 10

Effect of substances on capillary permeability

DILATE CAPILLARIES	LEAVE CAPILLARIES UNAFFECTED
Nutrient broth	Blood serum
Difco peptone	Cerebrospinal fluid
Savita broth without peptone	Egg albumin
Histamine	Liver extract
Testicular extract	Kidney extract
Leech extract	Histidine
Saliva	Arginine
Saline extract of feces	Cysteine
Blood group substances	Inositol
	Biotin

Witebsky (54) and produced commercially, as well as a blood group A substance prepared in a chemically pure form by Kabat *et al* (31, 3), had a strong effect on the capillaries Biological and clinical implications of this observation will not be discussed here

As was to be expected, histamine has a very strong effect on capillary permeability Even in a dilution of 1/100,000 this effect is still marked We have shown that our method can be used to study the effect of antihistaminic drugs quantitatively (51)

From the physiological point of view the quantitative aspect of the problem is of particular interest It has been shown in table 7 that broth increases the permeability of the capillaries of the skin to antitoxin about twenty-two times It will be recalled that according to table 1, the normal coefficient, K, of the distribution of antitoxin between blood and skin was likewise twenty-two It can easily be shown that the identity of these two figures is no coincidence Assume that the equation for the normal capillaries reads $Ai/Ad = KP/V$ After artificial increase of the capillary permeability, the equation takes the form

$Ai'/Ad = K'P/V$ Hence, $Ai/Ai' = K/K'$ Now it is obvious that maximal permeability of the capillaries will be reached when the concentration of antitoxin in blood and tissue have become identical In this case $K' = 1$ and $Ai/Ai' = K$

For the theory of capillary permeability, it is a remarkable fact that substances which have no apparent destructive properties for cell structures remove all barriers between blood and tissue in a reversible manner

The avidities of toxins for tissues and antitoxins

In a preceding section, it has been shown that in skin tests with diphtheria toxin, the Ai/Ad ratio can be defined by the equation $Ai/Ad = KP/V$, and that in this case the experimentally determined ratio is 22,000 This simple relation was shown to be due to the fact that in the case of diphtheria toxin, the Ai/Ad ratio is determined exclusively by the distribution of antitoxin between blood and tissue, and this in turn was shown to be a consequence of the very rapid reaction between toxin and antitoxin Diphtheria toxin has no opportunity to react with the tissue in the presence of antitoxin

It would be unjustified, however, to assume that the same simple relations hold true for all pathogenic agents In contrast to the immunity reactions *in vitro*, the immunity reactions *in vivo* take place in the presence of susceptible tissue cells and the interaction between pathogenic agents, antibodies, and tissues may have a marked effect on the Ai/Ad ratio This section will deal with these more complicated matters

As is well known the reaction between tetanus toxin and its antitoxin is much slower than the reaction between diphtheria toxin and antitoxin The possibility must be considered, therefore, that a reaction between toxin and tissue might interfere with the reaction between toxin and antitoxin and that this interference might be reflected in the value of Ai/Ad An attempt to approach this problem experimentally brought forth a very unexpected observation (19) It was found that the Ai/Ad ratios for individual tetanus toxins showed tremendous differences In a summarized form, these results are recorded in table 11 which shows that the Ai/Ad ratios vary over a range from 100 to 12,500 The Ad values do not differ significantly But the Ai values for toxins 1175H and 641B are 100 times higher than that for toxin 388 The differences in the ratios, therefore, are predominantly due to differences in the Ai values In principle, the same observation was made in indirect intramuscular tests with tetanus toxin (21) Table 12 shows that the Ai value for toxin 641B is approximately 62 times higher than that for toxin B

While in the direct test 20 lethal doses of all tetanus toxins are neutralized by approximately the same amounts of antitoxin, there exist qualitative differences between individual tetanus toxins which could be discovered only with the aid of the indirect test The next problem was the experimental analysis of these differences

In the indirect intracerebral test we are dealing with immunity reactions *in vivo* Two reactions take place at the same time in the central nervous system

namely a reaction between toxin and antitoxin and a reaction between toxin and nerve tissue. The result, therefore, will depend not only on the concentrations of the two components but also on the velocities of the reactions between toxin and antitoxin, and toxin and tissue, respectively. A high avidity of the toxin for tissue will increase the antitoxin requirements, while a high avidity of the toxin for antitoxin will decrease them.

TABLE 11

Ai, Ad and Ai/Ad values for 7 tetanus toxins

In the indirect test, serial dilutions of tetanus antitoxin were injected intravenously into guinea pigs weighing 250 g. Immediately afterwards, 20 MLD of the individual toxins were given intracerebrally. In the direct test, a constant dose of toxin and serial dilutions of antitoxin were mixed *in vitro*, and 0.1 ml of the mixtures containing 20 MLD were injected intracerebrally.

TOXIN SAMPLE	*Ai* (ml)	*Ad* (ml)	*Ai/Ad*
388	0.002	—	—
47	0.008	—	—
64	0.0031	0.000031	100
103	0.0062	0.000031	200
Lilly	0.04	0.000085	470
1175 H	0.2	0.000016	12,500
641 B	0.2	0.000016	12,500

TABLE 12

Indirect intramuscular tests with 9 tetanus toxins

Serial dilutions of antitoxin 387 (1200 units per ml) were given intravenously. Intramuscular test dose of each toxin was 20 MLD.

TOXIN SAMPLE	*Ai* (ml)	TOXIN SAMPLE	*Ai* (ml)
641 B	0.05	J	0.0062
1556	0.025	K	0.0031
1346	0.0125	L	0.0031
1375	0.0125	B	0.0008
H	0.0062		

Under certain experimental conditions, the velocity of the reaction between toxin and antitoxin can be measured by the velocity of flocculation in the Ramon test. Of course differences in the potencies of the toxins must be eliminated by proper dilutions. After doing this, the flocculation rates were found (21) to be approximately the same for all tetanus toxins examined. Consequently, the differences in the *Ai* values cannot be explained by differences in the avidities of the toxin for antitoxin.

The following experiments (21) will show that the differences in the *Ai* values are determined by differences in the avidities of the toxins for nerve tissue. The possibility of proving this matter experimentally is due to the fortunate

circumstance that yet another phenomenon is determined by the avidity of the toxin for nerve tissue As mentioned in an earlier section, the antitoxin requirements increase with the interval between the injection of toxin and the subsequent injection of antitoxin This has been explained by assuming that the combination between toxin and tissue undergoes a gradual increase in firmness and that, consequently, it becomes increasingly difficult for the antitoxin to dislodge the toxin from the tissue If this explanation is accepted, it implies

TABLE 13

Increase of antitoxin requirements associated with the interval between injection of tetanus toxin and antitoxin

Two identical experiments were performed with *toxins 64 and 641 B* In both, 20 lethal doses of toxin were injected intracerebrally In series A, serial dilutions of antitoxin were given intracerebrally immediately after the toxin In series B, the antitoxin was given 3 hours later

ANTITOXIN (ML)	TOXIN 64 MLD = 0.1 ML, 1/50 TEST DOSE = 0 1 ML, 1/2.5 (20 MLD) RESULTS*		TOXIN 641B MLD = 0.1 ML, 1/1600 TEST DOSE = 0 1 ML, 1/80 (20 MLD) RESULTS*	
	A	B	A	B
0 016	—	—	—	S,S
0 008	—	—	—	S,3
0 004	—	S,S	—	S,3
0 002	—	S,S	—	2,2
0 001	—	S,7	—	1,2
0 0005	—	6,8	—	1,1
0 00025	—	4,7	—	—
0 000125	S,S,S	2,7	—	—
0 000062	S,5	2,2	S,S	—
0 000031	4,4	—	S,6	—
0 000016	2,2	—	2,7	—
0 000008	2,4	—	1,2	—
0 000004	—		1,2	—
Protecting dose (ml)	0 000125	0 002	0 000062	0 016
B A	16 1		260 1	
A_t	0 003		0 2 ml	

* — = not done, S = survived, numerals indicate day of death after injection

that the increase in the antitoxin requirement should be determined by the avidity of the toxin for nerve tissue If the A_t value were likewise determined by this avidity, there should be positive correlation between the A_t values and the increase in the antitoxin requirements associated with the interval between the injections of toxin and antitoxin

To test the validity of these conclusions an experiment was performed with toxin 64 (A_t = 0 003 ml) and toxin 641B (A_t = 0 2 ml) It will be seen from table 13 that the increase in the antitoxin requirements owing to the interval between the injection of toxin and antitoxin is indeed correlated with the

values The increase was 16 1 for toxin 64 with an *Ai* value of 0 003 ml, but 260 1 for toxin 641B with an *Ai* value of 0 2 ml Since differences in the avidities of the toxins for antitoxin were excluded by previous experiments, these results can be interpreted only as indicating that the differences in the *Ai* values are determined by the differences in the avidities of the tetanus toxins for nerve tissue

These results illustrate the usefulness of studying immunity reactions *in vivo* The hitherto unknown qualitative differences between individual tetanus toxins could be discovered only with the aid of the indirect test, whereas they passed unnoticed in the direct test [3] In several respects, the results should be of some practical significance The experiments show that tetanus toxins with high avidities for nerve tissue are practically resistant to antitoxin under conditions as they prevail in the natural disease Our results show further that in the case of tetanus toxin, the standardization of the antitoxin is of limited value The standardization methods are based on the assumption that the antitoxin requirements are determined by the combining power of the test dose of toxin In the indirect test and in the natural disease, the antitoxin requirements are to a large extent dependent on the avidity of the toxin for nerve tissue Since this avidity varies from case to case and is unknown to the clinician, there is little hope that any standardization method will be able to overcome this difficulty

In the preceding paragraphs, it has been shown that in the case of tetanus toxin, the *Ai/Ad* ratio, and in a broader sense, the curative effect of antitoxin is strongly affected by the avidity of the toxin for nerve tissue It was shown at the same time that individual tetanus toxins do not vary in their avidities for antitoxin It will now be shown that various samples of antitoxin may vary in their avidities for the same toxin, and in their curative values Earlier investigations did not make use of the method of the *Ai/Ad* ratio, but they are closely related to our subject because they deal with the problem of immunity reactions *in vivo* Roux was the first to observe that the curative values of antidiphtheric sera did not always run parallel to their potencies expressed in terms of units of antitoxin This question was investigated on a much larger scale by Kraus and Schwooner (34) and by Kraus and Baecher (32) They first injected toxin into guinea pigs and then antitoxin after varying intervals In conformity with the results of Roux, they found no parallelism between the curative powers of the sera and their potency in terms of units of antitoxin Kraus advanced the theory that the curative value of antitoxic sera is dependent not only on their strength as determined by the method of Ehrlich, but also on the avidities of the antitoxins for toxin These experiments led to a lively but inconclusive controversy between Kraus and the Ehrlich school

[3] After it had been found that broth strongly increases the effect of antitoxin in the indirect test, the possibility was considered that the differences in the *Ai* values of the individual toxins might be due to differences in the broth contents of the test doses In this case, the *Ai* value should be determined by the potency of the toxins However, in a large number of experiments no correlation was found between these two quantities The effect of broth on capillary permeability diminishes rapidly upon dilution and this is probably the reason why it has little influence on the *Ai* value

The question was finally settled by the experimental investigations of Madsen and Schmidt (36, 37). They observed that mixtures of diphtheria toxin and some antitoxins were neutral when injected subcutaneously into guinea pigs but were highly toxic when injected intravenously into rabbits. Other antitoxic sera did not show this phenomenon. Madsen and Schmidt concluded that sera of the first type react slowly with toxin while sera of the second type react rapidly. This explanation was supported by the observation that sera of the first type usually precipitated toxin more slowly in the Ramon test than did sera of the second type. Finally it was found that sera of the second type were therapeutically more potent than sera of the first type. These investigations, therefore, can be considered as conclusive evidence for the theory of Kraus.

Glenny and his coworkers (24, 25, 27) have tried to define the concept of avidity more precisely. They do not consider the rapidity of the reaction between toxin and antitoxin as essential. Of greater importance is the firmness of the combination between the two. The mixtures of toxin and some antitoxins dissociate readily upon dilution. For this reason a mixture of large amounts of toxin and antitoxin may be neutral while a tiny fraction of this mixture may be highly toxic. For example, Glenny and Barr (24) prepared a mixture of toxin and antitoxin which was neutral when injected intravenously into a rabbit in a volume of 10 ml, whereas, 0.001 to 0.5 ml was lethal. Glenny *et al.* have developed methods with the aid of which the avidities of antitoxins for toxin can, to some extent, be measured quantitatively. The reader may be referred to the discussion of this work by Wilson and Miles (53).

The Ai/Ad ratio and the mechanism of immunity in neurotropic virus diseases

In the field of neurotropic virus diseases, the *Ai* and *Ad* values stand for familiar concepts. The *Ad* value represents the neutralizing amount of antiserum in the customary neutralization test. The *Ai* value measures the minimal amount of antiserum which provides passive protection against the intracerebral injection of the virus. The *Ai/Ad* ratio, therefore, is the ratio between the passively protecting and the neutralizing dose of antiserum.

According to the indirect intracerebral tests with diphtheria toxin discussed earlier, the *Ai/Ad* ratio should be 6200:1 for all neurotropic viruses, if this ratio were exclusively determined by the distribution of the antibody between blood and brain. It can be shown, however, that the *Ai/Ad* ratios for some of the neurotropic viruses differ widely from the "standard" value of 6200:1, and further, that the *Ai/Ad* ratios for the individual viruses differ widely among themselves.

The *Ai/Ad* ratio for the virus of rabies was determined (20) in the experiments recorded in table 14. As may be seen, 1 ml of undiluted serum did not protect guinea pigs passively against as little as 10 lethal doses. It is impossible, therefore, to determine an *Ai/Ad* ratio. Since, however, the *Ad* value was 0.1 ml, 1/64, the ratio was at least 1240 and consequently of the same order as that for diphtheria toxin.

In the experiments recorded in table 15, the *Ai/Ad* ratio was determined for

the virus of Eastern equine encephalomyelitis (20) The results indicate that the Ai/Ad ratio for the virus of equine encephalomyelitis is 5 1 This extremely low value is totally at variance with the "standard" value of 6200 1 and ob-

TABLE 14

Determination of the Ai and Ad values for the virus of rabies in guinea pigs weighing 200 g

In the indirect test, serial dilutions of antiserum were given intravenously Immediately afterwards 0 1 ml of a 1/100,000 dilution of rabies virus (10 MLD) was injected intracerebrally In the direct test, 0 3 ml of a virus dilution 1/50,000 and 0 3 ml of serial serum dilutions were mixed *in vitro* and 0 1 ml of each mixture injected intracerebrally without incubation

DETERMINATION OF *Ai*		DETERMINATION OF *Ad*	
Serum dilution (1 ml dose)	Survivals*	Serum dilution (0 1 ml dose)	Survivals*
Undiluted	9,9	1/4	S,S
1/2	9,9	1/8	S,S
1/4	9,9	1/16	S,S
1/8	S,S	1/32	S,S
		1/64	S,S
		1/128	9,S
		1/256	9,9

* S = survived, numerals indicate day of death

TABLE 15

Determination of the Ai and Ad values for the virus of eastern equine encephalomyelitis

In the indirect test, serial dilutions of antiserum were given intravenously while immediately afterwards 0 1 ml of a 1/100 suspension of the virus (160 MLD) was injected intracerebrally In the direct test, 0 3 ml of 1/50 virus dilution and 0 3 ml of serial serum dilutions were mixed *in vitro*, and 0 1 ml of each mixture injected intracerebrally The experiments were carried out in guinea pigs weighing 200 g

DETERMINATION OF *Ai*		DETERMINATION OF *Ad*	
Antiserum (ml)	Survivals*	Antiserum (ml)	Survivals*
0 5	S,S	0 05	S,S
0 25	S,S	0 025	6,7
0 125	6,10	0 0125	8,10
0 062	6,7	0 0062	6,6
0 031	5,10	0 0031	4,6
0 016	4,4	0 0016	4,10
Ai = 0 25 ml		*Ad* = 0 05 ml	

* S = survived, numerals indicate day of death

viously calls for an explanation An important step in this direction was made by the fundamental investigation of Olitsky and Harford (42) These authors found that equine encephalomyelitis antiserum has a very low potency in the direct intracerebral test but is very potent in the intraperitoneal test in young

mice In confirmation of these results we have found that 0 1 ml of a 1/250 dilution of serum protected in the intraperitoneal test, whereas as much as 0 1 ml of a 1/4 dilution was required in the intracerebral test The low Ai/Ad ratio, therefore, is at least partly explained by the high Ad value The problem is thus reduced to an explanation of the relative inefficiency of equine encephalomyelitis antiserum in the direct intracerebral test

This problem has been discussed in the literature, and the plausible explanation has been advanced that in the intracerebral test the proximity of the brain interferes with the reaction between virus and antibody For reasons which will become apparent below, this explanation is unacceptable Our theory follows a different line of reasoning

Schaeffer and Muckenfuss (47, 48) and Friedemann, Zuger and Hollander (14) found independently that intracerebrally injected fluid always reaches the intraventricular fluid Even after injection of colored fluid directly into the exposed cerebral cortex, the substance of the brain remains uncolored The fluid either reaches the ventricles or runs out of the channel of injection into the subarachnoid space In the direct test, therefore, the reaction between virus and antibody can take place only in the cell-free cerebrospinal fluid It is tantamount to an *in vitro* reaction

In the last analysis, therefore, the difference in the Ai/Ad ratios for the viruses of rabies and equine encephalomyelitis must be due to some fundamental difference in the mechanism of the virus-antibody reaction *in vitro* It looks as if the virus of equine encephalomyelitis reacts very little, if at all, with its antibody in the cell-free spinal fluid, while the contrary holds true for the virus of rabies To test the validity of this hypothesis, direct intracerebral tests were performed with incubated and non-incubated mixtures (20) The results of these experiments are recorded in tables 16 and 17

As may be seen from table 16, the protecting dilution of antiserum in the non-incubated mixtures of rabies virus was 1/16 but in the incubated mixtures it was at least 1/8142 The potency, therefore, was at least 500 times higher in the incubated than in the non-incubated mixtures This experiment shows clearly that rabies virus and its antibody react with each other *in vitro*

On the other hand table 17 shows that the potency of equine encephalomyelitis antiserum is not at all increased by incubation Since the serum was found to be so potent in the intraperitoneal test, it is clear that the virus of equine encephalomyelitis does not react with its antibody *in vitro* unless the latter is diluted to less than 1/2

This fundamental difference between the two viruses is most interesting, but apparently it is no great help in understanding the mechanism of immunity in equine encephalomyelitis On the contrary, it poses a most difficult problem If equine encephalomyelitis virus does not react with its antibody *in vitro*, how is it possible that the serum protects passively against the intracerebral injection of the virus, and why is the Ai/Ad ratio exceptionally low?

The following considerations will show that the mechanism of the virus-antibody reaction in the indirect test must be different from that in the direct test

The protecting dose in the direct test was 0 05 ml If the mechanism of the reaction were the same in the direct and the indirect tests and if, consequently, the Ai/Ad ratio were exclusively determined by the distribution of the antibody

TABLE 16

Direct intracerebral tests with rabies virus in incubated and non incubated mixtures

0 1 ml of a 1/100,000 dilution (100 MLD) of rabies virus was mixed with serial dilution of antiserum In one series the mixtures were kept at room temperature for 24 hours In another series, virus and antiserum were kept separately at room temperature for 24 hours and mixed immediately before injection Mixtures were injected intracerebrally into guinea pigs weighing 200 g

SERUM DILUTION	SURVIVAL* FROM INCUBATED MIXTURES	SURVIVAL* FROM NON INCUBATED MIXTURES
1/16	S,S	S,S
1/32	S,S	S,11
1/64	S,S	S,10
1/128	S,S	S,9
1/256	S,S	9,9
1/512	S,S	9,9
1/1024	S,S	—
1/2048	S,S	—
1/4096	S,S	—
1/8192	S,S	—

* S = survived, numerals indicate day of death

TABLE 17

Direct intracerebral tests with the virus of equine encephalomyelitis in incubated and non-incubated mixtures

0 1 ml of equine encephalomyelitis virus in a dilution of 1/640 (100 MLD) was mixed with serial dilutions of antiserum As in table 16, the experiment was carried out with incubated and non-incubated mixtures in guinea pigs weighing 200 g

ANTISERUM	SURVIVALS* FROM INCUBATED MIXTURES	SURVIVALS* FROM NON INCUBATED MIXTURES
1/2	S,S	S,S
1/4	5,8	S,5
1/8	5,5	S,6
1/16	6,6	6,5
1/32	5,4	6,4
1/64	6,5	4,5
1/128	5,8	6,4

* S = survived, numerals indicate day of death

between blood and brain, the ratio would be the same as in the experiments with diphtheria toxin, namely 6200 1, and Ai would have a value of 320 ml Experimentally, however, an Ai value of 0 25 ml was found If we consider that in the direct test the virus-antibody reaction takes place in the cell-free spinal

fluid, while in the indirect test virus and antibody react with each other in the substance of the brain, we can hardly escape the conclusion that in the indirect test the tissue cells provide something that is necessary for the reaction between virus and antibody

Fortunately this is not an arbitrary hypothesis but fits in very well with the investigations of Sabin (46) on virus immunity Both Andrewes (1) and Sabin have shown that the viruses of vaccinia, pseudorabies, and virus B do not react with their respective sera *in vitro* and consequently, resemble in this respect, the virus of equine encephalomyelitis Sabin, however, found in carefully planned experiments that the above mentioned viruses are acted upon by their antibodies in the presence of susceptible tissue cells He treated these cells first with anti serum, washed them and then added the virus This procedure prevented the development of inclusion bodies which formed in cells treated with the virus alone Sabin assumes that the antibody coats the surface of the cells and thus prevents the viruses from entering them

Similar experiments with the virus of equine encephalomyelitis have not been performed It appears, however, very plausible to assume that the discrepancy between the inefficiency of the encephalomyelitis antiserum *in vitro* and its high efficiency in the animal body is explicable along similar lines as in the experiments with vaccinia virus, pseudorabies, and virus B

An experimental analysis of the *Ai/Ad* ratios of the viruses of rabies and equine encephalomyelitis has thus revealed the existence of two groups of neurotropic viruses with entirely different mechanisms of immunity

It will be of great interest to determine the distribution of other neurotropic viruses between these two groups Although, thus far, this problem has not been approached systematically, observations reported in the literature give some hints It has already been mentioned that the pseudorabies virus and B virus fail to combine with their antibodies *in vitro* and behave in this respect like equine encephalomyelitis virus More recently Lennette and Koprowski (35) have shown that the result obtained by Olitsky and Harford (42) for the virus of equine encephalomyelitis holds true for a number of other viruses In experiments with the viruses of Eastern and Western equine encephalomyelitis, Venezuelan encephalomyelitis, Western Nile disease, St Louis encephalomyelitis, Japanese encephalomyelitis and yellow fever, it was found that antibodies can be easily identified in the intraperitoneal test in young mice, while this was difficult or impossible in the intracerebral test It would appear that as far as the mechanism of immunity is concerned, all these viruses belong in one group It must be emphasized, however, that some of these viruses are closely related to each other

On the other hand, the virus of poliomyelitis seems to resemble the virus of rabies Olitsky and Cox (41), Harmon and Harkins (29), Gordon (28), and Schultz and Gebhardt (50) found difficulties in protecting rhesus monkeys passively against the intracerebral injection of poliomyelitis virus even by the intravenous administration of the largest amounts of a highly potent immune serum Since antibodies against the virus of poliomyelitis can be identified with

the aid of the direct intracerebral test in monkeys, the findings of the above mentioned authors seem to indicate that the Ai/Ad ratio for the virus of poliomyelitis is very high and resembles in this respect the virus of rabies

It would be premature to predict which type of virus is more frequently met Much more experimental material will be required to decide this question It need hardly be emphasized that our knowledge in this field is still very incomplete To characterize a neurotropic virus, it will be necessary to determine the following data the Ai/Ad ratio, the Ad value in the intracerebral test for incubated and non-incubated mixtures, determination of the antibody titer in the intracerebral test and the intraperitoneal test in young mice, and if technically possible, the decision of the question whether the antibody requires the cooperation of tissue cells Only when these questions can be answered for a sufficient number of neurotropic viruses, will it be possible to develop a well founded theory of immunity in neurotropic virus diseases The results reported in this review, however, indicate the trend along which further work is desirable

It has been seen in the preceding paragraphs that the mechanism of the virus-antibody reaction determines to a large extent the Ad value and therefore, indirectly the Ai/Ad ratio The question arises, whether, in addition, the mechanism of the virus-antibody reaction also influences the Ai value On the basis of the existing experimental evidence, it is impossible to rule out this possibility It would even be plausible to assume that an antibody which coats the cells might be more effective than an antibody which neutralizes the virus outside the cells Perhaps certain observations on passive immunity might become understandable along these lines We have mentioned how difficult it is to protect experimental animals passively against the intracerebral injection of the viruses of rabies and poliomyelitis On the other hand, the experiments reported in this review as well as the investigations of Cox and Olitsky (4), Howitt (30), Zichis and Shaughnessy (55), and Olitsky *et al* (43) show that passive protection is obtained very easily with the virus of equine encephalomyelitis Although the interpretation of these observations certainly calls for further experimental work, these considerations may be mentioned in order to show that further studies on immunity reactions *in vivo* and particularly on the Ai/Ad ratio might lead to new viewpoints in the field of virus immunity

Permeability of the cerebral capillaries to antibodies

For many years it has been the prevailing opinion that the cerebral capillaries are impermeable to antibodies This concept is at variance with our results obtained in the indirect intracerebral tests with tetanus toxin and the virus of equine encephalomyelitis The question of the permeability of the cerebral capillaries to antibodies, therefore, called for a special investigation

The concept of the impermeability of the so called blood-brain barrier to antibodies was based on the very low concentration of antibodies in the cerebrospinal fluid Since according to the older theories, antibodies were supposed to reach the central nervous system only by way of the choroid plexus, the almost complete absence of antibodies from the spinal fluid was considered as evidence that

antibodies do not reach the central nervous system at all. The difficulty in immunizing experimental animals passively against the virus of rabies or poliomyelitis was explained by some authors on this basis.

This argument is no longer significant, for it is now the consensus of opinion that the exchange of substances between blood and brain takes place directly through the walls of the cerebral capillaries (11, 52, 10). There are, however, other observations on record which were interpreted as indicating that at least in some species the cerebral capillaries are impermeable to antibodies. This concept goes back to old experiments of Roux and Borrel (45). They found that rabbits actively or passively immunized against tetanus toxin were not protected against the intracerebral injection of a single lethal dose of the toxin. Later these experiments were repeated by Descombey (5), and Mutermilch and Salamon (40) in guinea pigs with entirely different results. Guinea pigs immunized in the same way as rabbits withstood the intracerebral injection of as much as 20 lethal doses of tetanus toxin. From these results the French authors concluded that the cerebral capillaries are permeable to antibodies in the guinea pig but impermeable to them in the rabbit.

This explanation appears rather artificial. The experiments reported in this review suggested an entirely different explanation. In the first place it has been shown that the *Ai* value depends on the size of the experimental animal. For this reason alone the protecting dose of antitoxin in the indirect intracerebral test should be 10 times higher in the rabbit than in the guinea pig. To make results in the two species comparable, therefore, the *Ai* value should be divided by the plasma volume, *P*.

In the second place the *Ai* value, in contradistinction to the *Ai/Ad* ratio is dependent on the combining power of the test dose of toxin. This is of paramount importance if, as in the experiments of the French authors, the test dose is measured in terms of lethal doses. Since the guinea pig is much more susceptible to tetanus toxin than the rabbit, one lethal dose in the rabbit represents a much higher combining power than one lethal dose in the guinea pig and requires for its neutralization a much higher amount of antitoxin.

The correctness of these considerations could be demonstrated in a convincing manner by experiments with diphtheria toxin (14). The intracerebral lethal dose of diphtheria toxin, in contrast to tetanus toxin, is the same for the rabbit and the guinea pig. If *Ai/P* were actually determined by the combining power of the test dose, it should, therefore, have the same value in both animals. On the other hand, if the cerebral capillaries in the rabbit were impermeable to diphtheria antitoxin, the experiments with diphtheria toxin would be a replica of those with tetanus toxin. The experiment gave a clear cut answer to this question. As will be seen from table 18, in the experiment with diphtheria toxin, the *Ai/P* ratio had substantially the same value in the guinea pig and the rabbit, whereas in the experiment with tetanus toxin, the *Ai/P* value was 200 times higher in the rabbit than in the guinea pig.

There is another way to show that the cerebral capillaries are equally permeable to antitoxins in the rabbit and the guinea pig. If *Ai/P* is determined exclusively

by the combining power of the test dose of toxin it should have the same value in the rabbit and the guinea pig if, irrespective of the lethal dose, equal amounts of toxins are given to both animals The experiment recorded in table 19 (14) shows that the Ai/P values, although not identical, approach each other closely A complete agreement between theory and experiment can hardly be expected since as was shown previously the Ai values for tetanus toxins in the guinea pig

TABLE 18

Determination of Ai for 10 lethal intracerebral doses of tetanus and diphtheria toxins in the rabbit and the guinea pig

10 lethal doses of tetanus toxin 388, 0 25 ml for the rabbit, and 0 0025 ml for the guinea pig, 10 lethal doses of diphtheria toxin, 0 0025 ml for both the rabbit and the guinea pig

ANIMAL	TETANUS ANTITOXIN (ml)	SURVIVALS*	DIPHTHERIA ANTITOXIN (ml)	SURVIVALS*
Rabbit (2500 g)	2	S,1	3	S,S
	1	1,5	2	S,6
	0 5	2	1	4,4
			0 5	5,6
Guinea pig (250 g)	0 001	S	0 5	S,S
	0005	9	0 25	S,7
	00025	10	0 125	5,6
	0002	3,3		
	0001	3 6		

* S = survived, numerals indicate day of death

TABLE 19

Experiments with tetanus toxins A and C

The test dose was the same in the guinea pig and the rabbit (10 lethal rabbit doses) The Ai values were determined in the usual way

TOXIN	TEST DOSE OF TOXIN (ML)	Ai/P* (RABBIT)	Ai/P* (GUINEA PIG)
A	0 025	0 02	0 03
C	0 00625	0 02	0 05

* P = plasma volume

are not exclusively determined by the combining power of the test dose but also by the avidities of the individual toxins for nerve tissue

The clarification of this problem is instructive because it shows how dangerous it is to compare experiments in different animal species on the basis of the Ai values alone

SUMMARY

The investigations reviewed in this survey deal with a hitherto rather unexplored field of immunology The classical method of evaluating immune sera

consists of mixing pathogenic agent and immune serum in the test tube and injecting the mixture into the experimental animal. Under these conditions the reaction between the two components takes place almost exclusively outside the animal body and the experimental animal serves only as an indicator of the unneutralized pathogenic agent. These reactions are in reality *in vitro* reactions. When immune serum is given for therapeutic or prophylactic purposes, however, the reaction between pathogenic agent and antibody takes place exclusively within the animal body. It is these immunity reactions *in vivo* with which the present article deals.

It was the final goal of these investigations to elaborate or at least to lay the foundation for a rational dosage of immune sera. For this purpose an attempt was made to determine in the animal experiment the ratio between the therapeutic dose of immune sera and their neutralizing dose *in vitro*, and to investigate the mechanism which determines this ratio.

It has been known since the early days of immunology that the therapeutic dose is usually infinitely larger than the neutralizing dose but the reason for this difference was imperfectly understood. It was realized early in the course of these investigations that this difference is largely due to the fact that in the diseased human body the pathogenic agent is within the tissue while the antibody circulates in the blood separated from the pathogenic agent by the capillary wall. To simulate these conditions as closely as possible, serial dilutions of immune serum were injected intravenously while immediately afterwards a constant dose of the pathogenic agent was injected into some tissue (skin, brain or muscle). Experiments of this type were designated as indirect tests and the minimal neutralizing dose of immune serum as Ai. With some qualifications, Ai may be considered as representing the therapeutic dose.

The neutralizing dose of immune serum was determined by mixing serial dilutions of immune serum and a constant dose of pathogenic agent *in vitro* and injecting 0.1 ml of each mixture into the same tissue as in the indirect test. These experiments were designated as direct tests and the neutralizing dose of immune serum as Ad.

The ratio, Ai/Ad, between the therapeutic and the neutralizing doses has been determined for a number of pathogenic agents. It has been shown that the mechanism determining the Ai/Ad ratio in the case of diphtheria toxin is exclusively determined by the distribution of antitoxin between blood and tissue and can be expressed by the formula $Ai/Ad = KP/V$, where K is the coefficient of distribution of antitoxin between blood plasma and tissue, P the plasma volume, and V the volume of fluid injected into the tissue. In skin test experiments on rabbits weighing 2500 g, Ai/Ad was found to have a value of 22,000. In a child weighing 30 Kg, the ratio would be approximately 440,000. This figure gives an approximate idea of the ratio between the therapeutic and the neutralizing doses of antitoxin in the case of diphtheria. Actually this is a minimum value. As is well known the therapeutic dose increases with the interval between the onset of the disease and the administration of antitoxin.

According to the above distribution equation, the Ai/Ad ratio should be the

same for all pathogenic agents, but in the case of other pathogenic agents, conditions are not so simple. Pathogenic agent and antibody may react not only with each other but also with the tissue and accordingly the ratio may be very different from those found for diphtheria toxin. Particularly interesting and unexpected observations were made in the case of tetanus toxin. The *Ai/Ad* values were determined for a considerable number of tetanus toxins in indirect intracerebral and intramuscular tests. Not only were the results very different from those obtained with diphtheria toxin but the *Ai/Ad* ratios for different tetanus toxins differed among themselves very considerably. For some toxins the ratios were 100 times higher than for others. Experimental analysis of these observations showed that the *Ai/Ad* values were determined by the avidities of the various toxins for nerve tissue.

Extreme differences in *Ai/Ad* were found in experiments with neurotropic viruses. While the ratio was at least 1280 for the virus of rabies, it had the very low value of 5 for the virus of equine encephalomyelitis. A further experimental analysis of these results uncovered a fundamental difference in the mechanism of immunity in the case of these two viruses. While the virus of rabies readily combines with its antibody *in vitro* even when the immune serum is highly diluted, the virus of equine encephalomyelitis does not react with its antibody *in vitro* unless the antiserum is undiluted. Since in the direct test virus and antibody react with each other exclusively in the cell-free ventricular fluid, the neutralizing dose in the direct test must be very high for the virus of equine encephalomyelitis. The fact that the *Ai* value is not proportionally high shows that the mechanisms of immunity in the direct and the indirect tests must be different. In analogy with the investigations of Sabin on the viruses of vaccinia and pseudorabies, and on virus B, it has been assumed that the virus of equine encephalomyelitis is acted upon by its antibody only when the latter has been fixed by nerve cells.

In view of the complicated nature of immunity in neurotropic viruses it can hardly be predicted to what extent our results can be generalized for other neurotropic viruses. Further investigations are called for to determine whether there exists any general rule correlating the *Ai/Ad* ratios of viruses with their ability or inability to react with their antibodies *in vitro*. There are some indications in our experiments that active or passive immunization against the intracerebral injection of viruses may be easy if the antibody is fixed by nerve cells while it may be difficult if the antibody reacts with the virus directly. This question also will require more extensive investigation.

The skin tests with diphtheria toxin, and the intracerebral tests with tetanus toxin and neurotropic viruses, have in common that the reaction to the pathogenic agent is observed at the site of its injection. Indirect tests, however, can be carried out in such a way that the reaction to the pathogenic agent is observed in organs remote from the site of injection. A case in point is the indirect intramuscular test with diphtheria toxin where the dose of antitoxin was determined which protects the experimental animals against death. In the direct test toxin and antitoxin were injected intravenously. In these experiments the antitoxin requirements were approximately the same for intramuscularly and intravenously

injected toxin This is due to the fact that diphtheria toxin reaches the heart and the adrenals by way of the circulation It is irrelevant, therefore, whether the toxin is neutralized at the site of its injection or in the blood stream

Analogous experiments with tetanus toxin gave an entirely different result Intramuscularly injected toxin required for its neutralization up to 80 times more antitoxin than intravenously *injected toxin This is due to the fact that tetanus* toxin reaches the central nervous system by way of the peripheral nerves It is essential, therefore, that the intramuscularly injected toxin be neutralized at the site of its injection

Finally it has been shown that the method of the indirect test can be used in studies on capillary permeability For this purpose the substance under investigation is mixed with the test dose of toxin in the indirect test while the antitoxin is given intravenously as usual An increase in capillary permeability is indicated by the enhanced neutralizing effect of the antitoxin With the aid of this convenient method, the effect of a large number of substances has been studied It is remarkable that most of the substances which affect the capillaries at all, increase their permeability to such an extent that the concentration of antitoxin on both sides of the capillary wall becomes identical

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